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(54) Title: DNA ENCODING ACYLCOENZYME A: CHOLESTEROL ACYLTRANSFERASE AND USES THEREOF		
(57) Abstract This invention provides an isolated nucleic acid which encodes an acylcoenzyme A: cholesterol acyltransferase II or III. Specifically, this invention provides an isolated nucleic acid which encodes a human wildtype acylcoenzyme A: cholesterol acyltransferase II or III. This invention also provides various methods for inhibiting wildtype acylcoenzyme A: cholesterol acyltransferase II or III in a subject. This invention also provides a method for identifying a chemical compound which is capable of inhibiting acylcoenzyme A: cholesterol acyltransferase II or III and a pharmaceutical composition comprising of the chemical compound identified by the above-described method. This invention also provides a method of treating a subject who has atherosclerosis or hyperlipidemia.		

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DNA ENCODING ACYLCOENZYME A: CHOLESTEROL
ACYLTRANSFERASE AND USES THEREOF

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This application is a Continuation-In-Part of U.S. Serial No. 08/657,620, filed May 30, 1996, the content of which is incorporated by reference into this application.

10 Throughout this application, various publications are referenced by Arabic numerals. Full citations for these publications may be found listed at the end of the specification. The disclosures of these publications in their entireties are hereby incorporated by reference
15 into this application in order to more fully describe the state of the art as known to those skilled therein.

Background of the Invention

Cholesterol or related sterols, required for the
20 viability of eukaryotic cells, exist in the free form or as esters conjugated to fatty acids. The concentration of free sterol determines the fluidity of eukaryotic cell membranes, whereas esterified sterols cannot participate in membrane assembly. The esterification of
25 intracellular sterol, mediated in mammals by the membrane-bound enzyme, acylcoenzyme A: cholesterol acyltransferase, is thus a critical homeostatic determinant of membrane function (1, 2). For example, cholesterol depletion of the rough endoplasmic reticulum
30 (ER) relative to the smooth ER (3), may modulate protein translocation or membrane-associated transcriptional activators such as the Sterol Response Element Binding proteins (SREBP, 4). In addition, production of cholesterol ester (CE) by acylcoenzyme A: cholesterol
35 acyltransferase in the rough ER may influence the transport of sterol between intracellular pools. Similar

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esterification activities have been observed in other eukaryotes such as plants and yeasts (5).

5 Elevations in acylcoenzyme A: cholesterol acyltransferase activity perturb several pathways that contribute to hyperlipidemia and atherosclerosis. Sterol esterification modifies the activity of the low density lipoprotein (LDL) receptor and alters serum lipoprotein composition to be pro-atherogenic (6, 7). It may also be
10 a rate limiting step in intestinal sterol absorption (8). Furthermore, CE deposition in the arterial wall is an important initial step in atherogenesis (9). The understanding of the acylcoenzyme A: cholesterol acyltransferase reaction has been hampered by the
15 difficulty of biochemical purification and by a poor grasp of the relevant genetic determinants. A human acylcoenzyme A: cholesterol acyltransferase I gene from macrophages was identified by complementation of Chinese Hamster Ovary cell lines deficient in acylcoenzyme A:
20 cholesterol acyltransferase activity (10) and was functionally expressed in insect cells devoid of endogenous activity (11).

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Summary of the Invention

This invention provides an isolated nucleic acid which encodes an acylcoenzyme A: cholesterol acyltransferase II
5 or an acylcoenzyme A: cholesterol acyltransferase III.

This invention also provides a vector which includes the isolated nucleic acid which encodes an acylcoenzyme A: cholesterol acyltransferase II or an acylcoenzyme A:
10 cholesterol acyltransferase III and a host vector system which includes a vector.

This invention also provides a method of producing a polypeptide which comprises growing such host vector
15 system of claim 14 under suitable conditions permitting production of the polypeptide and recovering the polypeptide so produced. This invention also provides a purified wildtype acylcoenzyme A: cholesterol acyltransferase II or an acylcoenzyme A: cholesterol
20 acyltransferase III.

This invention also provides an oligonucleotide of at least 15 nucleotides capable of specifically hybridizing with a unique sequence of nucleotides present within a
25 nucleic acid which encodes a wildtype acylcoenzyme A: cholesterol acyltransferase II or an acylcoenzyme A: cholesterol acyltransferase III without hybridizing to a nucleic acid which encodes a mutant acylcoenzyme A: cholesterol acyltransferase II or an acylcoenzyme A:
30 cholesterol acyltransferase III. This invention also provides an oligonucleotide of at least 15 nucleotides capable of specifically hybridizing with a unique sequence of nucleotides present within the nucleic acid which encodes a mutant acylcoenzyme A: cholesterol
35 acyltransferase II or an acylcoenzyme A: cholesterol

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acyltransferase III without hybridizing to a nucleic acid which encodes a wildtype acylcoenzyme A: cholesterol acyltransferase II or an acylcoenzyme A: cholesterol acyltransferase III.

5

This invention also provides a method for determining whether a subject known to have an imbalance in sterol levels has the imbalance due to a defect in esterification of sterol and for treating a subject who
10 has an imbalance in sterol levels due to a defect in esterification of sterol.

This invention also provides methods for inhibiting wildtype acylcoenzyme A: cholesterol acyltransferase II
15 or an acylcoenzyme A: cholesterol acyltransferase III in a subject.

This invention also provides a method for identifying a chemical compound which is capable of inhibiting
20 acylcoenzyme A: cholesterol acyltransferase II or an acylcoenzyme A: cholesterol acyltransferase III in a subject and a pharmaceutical composition comprising of the chemical compound so identified.

25 This invention also provides a transgenic, nonhuman mammal comprising the isolated nucleic acid which encodes acylcoenzyme A: cholesterol acyltransferase II or an acylcoenzyme A: cholesterol acyltransferase III.

30

Brief Description of the Figures

Abbreviations: The amino acid residues are abbreviated as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr. CON: consensus sequence.

Figures 1A and 1B. Protein sequence alignments predicted from candidate genes for the human acylcoenzyme A: cholesterol acyltransferase gene I, the yeast homologs, *acylcoenzyme A: cholesterol acyltransferase-related enzyme I* and *acylcoenzyme A: cholesterol acyltransferase-related enzyme II*, and a consensus sequence of all three sequences.

Identical residues between all the sequences are in bold face. Residues of the candidate leucine zipper heptad motif are italicized. Potential transmembrane domains were identified at residues 132 to 155 and 460 to 483; 186 to 202 and 406 to 421; and 215 to 231 and 439 to 451, for human acylcoenzyme A: cholesterol acyltransferase (Sequence I.D. No.: 2), *acylcoenzyme A: cholesterol acyltransferase-related enzyme I* (Sequence I.D. No.: 4) and *acylcoenzyme A: cholesterol acyltransferase-related enzyme II* (Sequence I.D. No.: 6), respectively. The firefly luciferase signature sequences identified in human acylcoenzyme A: cholesterol acyltransferase I (10) were not conserved in the yeast genes. CON (Sequence I.D. No.: 13) denotes the consensus sequence between the sequences of human acylcoenzyme A: cholesterol acyltransferase, *acylcoenzyme A:*

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- cholesterol acyltransferase-related enzyme I and acylcoenzyme A: cholesterol acyltransferase-related enzyme II. R07932 denotes the partial sequence of another human acylcoenzyme A: cholesterol acyltransferase candidate cDNA (residues 500 to 600) (Sequence I.D. No.: 14). The asterisks indicate the residues in R07932 identical to those of the other sequences.
- 5
- 10 1A. Alignment of amino acid residues 1-362 of acylcoenzyme A: cholesterol acyltransferase-related enzyme I and the identical residues in acylcoenzyme A: cholesterol acyltransferase-related enzyme II, human acylcoenzyme A: cholesterol acyltransferase and CON.
- 15
- 1B. Alignment of amino acid residues 363-611 of acylcoenzyme A: cholesterol acyltransferase-related enzyme I and the identical residues in acylcoenzyme A: cholesterol acyltransferase-related enzyme II, human acylcoenzyme A: cholesterol acyltransferase and CON.
- 20

25 **Figures 2A, 2B, 2C, 2D and 2E. Construction and analysis of acylcoenzyme A: cholesterol acyltransferase genes and deletion mutants.**

- 2A. The *are1* DNA deletion. The schematic depicts a fragment from yeast chromosome III in plasmid pH3(34). Strategic restriction endonucleases are indicated (H, Hind III; B, Bam HI).
- 30 2B. The autoradiogram depicts Bam HI digested DNA from wild-type or disrupted diploid strains probed with the 2993-bp Bam-HI fragment. This produced a fragment corresponding to the wild-type acylcoenzyme A: cholesterol

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acyltransferase-related enzyme I locus and a 1984-bp fragment characterizing the *are1Δ* NA allele. The diploid is heterozygous for the acylcoenzyme A: cholesterol acyltransferase-related enzyme I deletion.

5
2C. Reduced stringency hybridization of yeast genomic DNA with acylcoenzyme A: cholesterol acyltransferase-related enzyme I coding sequences. Genomic DNA from wild-type or
10 *ARE1/are1Δ*NA diploids were reprobed with an Nhe I-Avr II fragment corresponding to the acylcoenzyme A: cholesterol acyltransferase-related enzyme I open reading frame ("ORF"). Hybridizations and washes were performed at
15 60°C in the absence of formamide.

2D. The *are2Δ* deletion. In step 1, PCR amplifying oligonucleotides, KO-5' and KO-3' and a *LEU2* template were used to produce the selectable yeast gene flanked at the 5' and 3' ends by
20 acylcoenzyme A: cholesterol acyltransferase-related enzyme II. In step 2, this was used to direct homologous recombination at acylcoenzyme A: cholesterol acyltransferase-related enzyme II by transformation of a diploid strain and selection for leucine protrophy. In step 3,
25 integrants to acylcoenzyme A: cholesterol acyltransferase-related enzyme II were identified by a PCR reaction using oligonucleotides flanking *ARE2* (*are2*-5' and *are2*-3') and a 3' ampimer within *LEU2* (*L2*-3').
30

2E. A 999-bp fragment identifies *are2Δ*, as shown in the ethidium bromide stained agarose gel. The wild-type fragment (2206-bp) is also produced

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in the same reaction. Leucine prototrophic transformants with deletions of acylcoenzyme A: cholesterol acyltransferase-related enzyme II were obtained at a frequency of ~2%. M indicates the 50-2,000-bp ladder markers (Bio-Rad Laboratories).

Figures 3A and 3B. Fluorescent staining of triglyceride and sterol ester.

The cells were grown in YEPD to stationary phase, washed with deionized H₂O, and incubated with 1 µg/ml Nile Red (1 mg/ml in acetone). Fluorescent images were obtained with a BioRad MRC600 laser scanning confocal microscope (BioRad Microscience, Hercules, CA) on an inverted Zeiss Atiovert microscope (Zeiss, OberKochem, Germany) using 63X (NA1.4) Zeiss Plan-apo infinity corrected objective. Samples were illuminated with the 488nm line from an argon ion laser and the fluorescence was visualized with a 540nm dichroic mirror and 550nm long-pass emission filter. Staining of the cytoplasmic lipid droplets was sensitive to treatment with isopropanol, proving them to be lipid in nature.

3A. Wild-type cells.

3B. *are1ΔNAare2Δ* double mutant cells.

Figures 4A, 4B, 4C and 4D. Neutral lipid and sterol biosynthesis in ARE deletion mutants.

Strain genotypes are as described in the text; dpm/mg dry weight: disintegrations per minute per milligram of dry weight of cells.

4A. Triglyceride biosynthesis. Total lipids were

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extracted from cells grown in media containing ^3H -oleate and analyzed by thin-layer chromatography.

4B. Sterol ester biosynthesis. Total lipids were extracted from cells grown in media containing ^3H -oleate and analyzed by thin-layer chromatography.

4C. Sterol ester biosynthesis in wild-type and mutant cells transformed with vector control (black box) or acylcoenzyme A: cholesterol acyltransferase-related enzyme I over-expression plasmids, YEp3-16 (increased copy number, shaded box) and pADH5-36 (transcription from the ADH promoter, open boxes). Cells were grown in selective media to maintain the acylcoenzyme A: cholesterol acyltransferase-related enzyme I expression plasmids. Lipids were labeled, extracted and analyzed as above.

4D. Sterol biosynthesis in acylcoenzyme A: cholesterol acyltransferase-related enzyme deletion mutants. Lipids were labeled in synthetic complete media containing $[1-^{14}\text{C}]$ acetate, saponified and extracted with hexane and subjected to thin layer chromatography analysis. The data is representative of three separate experiments and expressed as the ratio of incorporation into sterols to incorporation into fatty acids.

Figures 5A, 5B, 5C, 5D, 5E and 5F. The nucleic acid and amino acid or predicted amino acid sequences.

5A-1 - 5A-3.

The nucleic acid sequence of human acylcoenzyme A: cholesterol acyltransferase I designated

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Sequence ID No.: 1. The amino acid sequence of human acylcoenzyme A: cholesterol acyltransferase I designated Sequence ID No.: 2.

- 5 5A-1. Nucleic acid sequence of human acylcoenzyme A: cholesterol acyltransferase I from nucleic acid bases 1-1624. Amino acid sequence of human acylcoenzyme A: cholesterol acyltransferase I from amino acid residues 1-76.
- 10 5A-2. Nucleic acid sequence of human acylcoenzyme A: cholesterol acyltransferase I from nucleic acid bases 1625-2524. Amino acid sequence of human acylcoenzyme A: cholesterol acyltransferase I from amino acid residues 77-376.
- 15 5A-3. Nucleic acid sequence of human acylcoenzyme A: cholesterol acyltransferase I from nucleic acid bases 2525-3649. Amino acid sequence of human acylcoenzyme A: cholesterol acyltransferase I from amino acid residues 377-551.
- 20
- 25

5B-1 - 5B-3.

30 The nucleic acid sequence of yeast acylcoenzyme A: cholesterol acyltransferase-related enzyme I designated Sequence ID No.: 3. The amino acid sequence of yeast acylcoenzyme A: cholesterol acyltransferase-related enzyme I designated Sequence ID No.: 4.

- 35 5B-1. Nucleic acid sequence of acylcoenzyme A: cholesterol acyltransferase-related enzyme I from nucleic acid

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bases 1-1289. Amino acid sequence of acylcoenzyme A: cholesterol acyltransferase-related enzyme I from amino acid residues 1-209.

5

5B-2.

Nucleic acid sequence of acylcoenzyme A: cholesterol acyltransferase-related enzyme I from nucleic acid bases 1290-2114. Amino acid sequence of acylcoenzyme A: cholesterol acyltransferase-related enzyme I from amino acid residues 210-484.

10

5B-3.

Nucleic acid sequence of acylcoenzyme A: cholesterol acyltransferase-related enzyme I from nucleic acid bases 2115-2601. Amino acid sequence of acylcoenzyme A: cholesterol acyltransferase-related enzyme I from amino acid residues 485-611.

15

5C-1 - 5C-3.

20

The nucleic acid sequence of yeast acylcoenzyme A: cholesterol acyltransferase-related enzyme II designated Sequence ID No.: 5. The amino acid sequence of yeast acylcoenzyme A: cholesterol acyltransferase-related enzyme II designated Sequence ID No.: 6.

25

5C-1.

Nucleic acid sequence of acylcoenzyme A: cholesterol acyltransferase-related enzyme II from nucleic acid bases 1-1061. Amino acid sequence of acylcoenzyme A: cholesterol acyltransferase-related enzyme II from amino acid residues 1-238.

30

5C-2.

Nucleic acid sequence of acylcoenzyme A: cholesterol acyltransferase-related enzyme II from nucleic acid

35

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bases 1062-1961. Amino acid sequence of acylcoenzyme A: cholesterol acyltransferase-related enzyme II from amino acid residues 239-538.

5 5C-3. Nucleic acid sequence of acylcoenzyme A: cholesterol acyltransferase-related enzyme II from nucleic acid bases 1962-2421. Amino acid sequence of acylcoenzyme A: cholesterol acyltransferase-related enzyme II
10 from amino acid residues 539-643.

5D. The nucleic acid sequence of mouse acylcoenzyme A: cholesterol acyltransferase II designated
15 Sequence ID No.: 11. The amino acid sequence of mouse acylcoenzyme A: cholesterol acyltransferase II designated Sequence ID No.: 12.

20 **Figure 6A. A restriction map of the expression vector YepAB-ACAT2.**

Figure 6B and 6C. Expression of human macrophage ACAT in pRS426GP.

25 6B. The ACAT open reading frame was inserted at the NotI and SacI sites, downstream of the promoter of the *GAL1/10* gene (*GAL1/10p*) as described in the text to produce pRS426-ACAT. *URA3* and *Amp^r* denote
30 selectable markers for yeast and *E. coli* respectively. The yeast and bacterial origins of replication (*2μm* and *ori*, respectively) are indicated.

6C. Immunoblot of human ACAT in protein

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5 extracts from cells transformed with pRS426-ACAT. Double mutant cells (*are1⁻ are2⁻*), transformed with pRS426-ACAT (hACAT) or with pRS426GP (vector), were induced by growth in galactose. Proteins were analyzed by immunoblotting. Equivalent amounts of protein extracts from mouse adrenal cells were loaded for comparison. Molecular weight reference markers (BioRad) are indicated (M). The arrow indicates the position of the DM10 immunoreactive product in extracts from murine adrenals. The expressed form of hACAT in yeast is of coincident mobility.

10
15 **Figures 7A and 7B. Multiple human tissue Northern analysis of poly (A)⁺ RNAs probed with ³²P-labeled cDNA C1.**

- 20 7A. Tissue specific expression of wildtype human acylcoenzyme A: cholesterol acyltransferase II using a wildtype acylcoenzyme A: cholesterol acyltransferase II specific probe.
- 25 7B. Tissue specific expression of wildtype human acylcoenzyme A: cholesterol acyltransferase I using a wildtype acylcoenzyme A: cholesterol acyltransferase I specific probe.

Figure 8A, 8B, 8C and 8D. Tissue specific expression of ARGP1 and hACAT.

- 30 8A and 8B. Multiple tissue Northern (Clontech) with indicated samples were probed with an ARGP1 specific probe as described in the text.
- 8C and 8D. The same blots were also analyzed using a hACAT specific probe. The first panel is

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identical to that published by Chang et al (8). The second panel is the same blot as in A and B, probed with the ACAT cDNA 1600 bp probe.

5

Figure 9. Fetal Tissue specific expression of AGRP2.

Multiple tissue Northern blots of fetal tissue (Clontech) with indicated samples, were probed with an AGRP2 specific probe as described in the text.

10

Figure 10. Cultured cell expression of AGRP1.

RNA samples from HepG2 and CV1 were reverse transcribed and PCR amplified as described in the text. P indicate a plasmid template control. The blank lanes represent water or no RT controls.

15

Figure 11. Sequence comparison of human ACAT and AGRP1

Figure 12. Sequence comparison of human ACAT and AGRP2

20

Figure 13. Phylogenetic Comparisons of ACAT like molecules.

The sequences shown were identified in genome databases and aligned based on protein sequence using GCG Inc software (pileup). They were subsequently arranged to their sequence conservation to determine approximate evolutionary relatedness.

25

Figure 14. Conserved motifs in ACAT related gene products.

30

Figure 15A and 15B. Nucleotide and predicted protein sequence of AGRP1.

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Figure 16. Nucleotide and predicted protein sequence of
ARGP2.

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Detailed Description of the Invention

Throughout this application, references to specific nucleotides are to nucleotides present on the coding strand of the nucleic acid. The following standard abbreviations are used throughout the specification to indicate specific nucleotides:

	C=cytosine	A=adenosine
10	T=thymidine	G=guanosine

A "gene" means a nucleic acid molecule, the sequence of which includes all the information required for the normal regulated production of a particular protein, including the structural coding sequence, promoters and enhancers.

The nucleic acids or oligonucleotides of the subject invention also include nucleic acids or oligonucleotides coding for polypeptide analogs, fragments or derivatives which differ from naturally-occurring forms in terms of the identity or location of one or more amino acid residues (deletion analogs containing less than all of the residues specified for the protein, substitution analogs wherein one or more residues specified are replaced by other residues and addition analogs where in one or more amino acid residues is added to a terminal or medial portion of the polypeptides) and which share some or all properties of naturally-occurring forms. These nucleic acids or oligonucleotides include: the incorporation of codons "preferred" for expression by selected non-mammalian hosts; the provision of sites for cleavage by restriction endonuclease enzymes; and the provision of additional initial, terminal or intermediate DNA sequences that facilitate construction of readily

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expressed vectors.

5 The nucleic acids and oligonucleotides described and
claimed herein are useful for the information which they
provide concerning the amino acid sequence of the
polypeptide and as products for the large scale synthesis
of the polypeptide by a variety of recombinant
techniques. The molecule is useful for generating new
cloning and expression vectors, transformed and
10 transfected prokaryotic and eukaryotic host cells, and
new and useful methods for cultured growth of such host
cells capable of expression of the polypeptide and
related products.

15 An isolated nucleic acid which encodes an acylcoenzyme A:
cholesterol acyltransferase II. This isolated nucleic
acid may be DNA or RNA, specifically cDNA or genomic DNA.
Specifically, the isolated nucleic acid has the sequence
designated Seq. I.D. No.: 7. The isolated nucleic acid
20 encodes a human wildtype acylcoenzyme A: cholesterol
acyltransferase II having substantially the same amino
acid sequence as the sequence designated Seq. I.D. No.:
8. Specifically the isolated nucleic acid has the
sequence designated Seq. I.D. No.: 11. The isolated
25 nucleic acid encodes a mouse wildtype acylcoenzyme A:
cholesterol acyltransferase II having substantially the
same amino acid sequence as the sequence designated Seq.
I.D. No.: 12. Further, the isolated nucleic acid of
encodes a mutant acylcoenzyme A: cholesterol
30 acyltransferase II.

An isolated nucleic acid which encodes an acylcoenzyme A:
cholesterol acyltransferase III. This isolated nucleic
acid may be DNA or RNA, specifically cDNA or genomic DNA.
35 Specifically, the isolated nucleic acid has the sequence

as set forth in Fig. 16. The isolated nucleic acid encodes a human wildtype acylcoenzyme A: cholesterol acyltransferase III having substantially the same amino acid sequence as set forth in Fig. 16. Further, the
5 isolated nucleic acid of encodes a mutant acylcoenzyme A: cholesterol acyltransferase III.

As used in this application, "acylcoenzyme A: cholesterol acyltransferase III" means and includes any polypeptide
10 having acylcoenzyme A: cholesterol acyltransferase III activity and having an amino acid sequence homologous to the amino acid sequence of human acylcoenzyme A: cholesterol acyltransferase II (the sequence of which is set forth in Fig. 15). Thus, this term includes any such
15 polypeptide whether naturally occurring and obtained by purification from natural sources or non-naturally occurring and obtained synthetically, e.g. by recombinant DNA procedures. Moreover, the term includes any such polypeptide whether its sequence is substantially the
20 same as, or identical to the sequence of any mammalian homolog of the human polypeptide, e.g. murine, bovine, porcine, etc. homologs. Additionally, the term includes mutants or other variants of any of the foregoing which retain at least some of the enzymatic activity of
25 nonmutants or nonvariants.

As used in this application, "acylcoenzyme A: cholesterol acyltransferase II" means and includes any polypeptide
30 having acylcoenzyme A: cholesterol acyltransferase II activity and having an amino acid sequence homologous to the amino acid sequence of human acylcoenzyme A: cholesterol acyltransferase III (the sequence of which is set forth in Fig. 16). Thus, this term includes any such polypeptide whether naturally occurring and obtained by
35 purification from natural sources or non-naturally

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occurring and obtained synthetically, e.g. by recombinant DNA procedures. Moreover, the term includes any such polypeptide whether its sequence is substantially the same as, or identical to the sequence of any mammalian homolog of the human polypeptide, e.g. murine, bovine, porcine, etc. homologs. Additionally, the term includes mutants or other variants of any of the foregoing which retain at least some of the enzymatic activity of nonmutants or nonvariants.

10

The invention also encompasses DNAs and cDNAs which encode amino acid sequences which differ from those of acylcoenzyme A: cholesterol acyltransferase II, but which do not produce phenotypic changes.

15

The invention also encompasses DNAs and cDNAs which encode amino acid sequences which differ from those of acylcoenzyme A: cholesterol acyltransferase III, but which do not produce phenotypic changes.

20

The nucleic acid of the subject invention also include nucleic acids that encode for polypeptide analogs, fragments or derivatives which differ from naturally-occurring forms in terms of the identity or location of one or more amino acid residues (including deletion analogs containing less than all of the residues specified for the protein, substitution analogs wherein one or more residues specified are replaced by other residues and addition analogs wherein one or more amino acid residues is added to a terminal or medial portion of the polypeptides) and which share some or all properties of the naturally-occurring forms.

25

30

35

The polypeptide of the subject invention also includes analogs, fragments or derivatives which differ from

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naturally-occurring forms, but having acylcoenzyme A: cholesterol acyltransferase activity.

5 This invention also provides a vector comprising an isolated nucleic acid encoding acylcoenzyme A: cholesterol acyltransferase II or III. The isolated nucleic acid of the vectors is operatively linked to a promoter of RNA transcription which maybe, or is identical to, a bacterial, yeast, insect or mammalian
10 promoter. The vector may be a plasmid, cosmid, yeast artificial chromosome (YAC), bacteriophage or eukaryotic viral DNA. Specifically, this invention provides a vector designated YepAB-ACAT2 (Figure 6).

15 Further other numerous vector backbones known in the art as useful for expressing proteins may be employed. Such vectors include but are not limited to: adenovirus, simian virus 40 (SV40), cytomegalovirus (CMV), mouse mammary tumor virus (MMTV), Moloney murine leukemia
20 virus, murine sarcoma virus, and Rous sarcoma virus, DNA delivery systems, i.e liposomes, and expression plasmid delivery systems.

25 This invention also provides a vector system for the production of a polypeptide which comprises the vector in a suitable host. Suitable host includes a cell which includes, but is not limited, prokaryotic or eukaryotic cells, e.g. bacterial cells (including gram positive cells), yeast cells, fungal cells, insect cells and
30 animal cells.

Suitable animal cells include, but are not limited to, HeLa cells, Cos cells, CV1 cells and various primary mammalian cells. Numerous mammalian cells may be used as
35 hosts, including, but not limited to, the mouse

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fibroblast cell NIH 3T3, CHO cells, Ltk⁻ cells, etc. Expression plasmids such as that described supra may be used to transfect mammalian cells by methods well known in the art such as calcium phosphate precipitation,
5 electroporation.

This invention also provides a method for producing a polypeptide (e.g. acylcoenzyme A: cholesterol acyltransferase) which comprises growing a host vector
10 system under suitable conditions permitting production of the polypeptide and recovering the polypeptide so produced. Methods of recovering polypeptides produced in such host vector systems are well-known in the art and typically include steps involving cell lysis,
15 solubilization and chromatography.

This invention also provides a method of obtaining a polypeptide in purified form which comprises: (a) introducing a vector, as described above, into a suitable
20 host cell; (b) culturing the resulting cell so as to produce the polypeptide; (c) recovering the polypeptide produced in step (b); and (d) purifying the polypeptide so recovered. As discussed above the vector may include a plasmid, cosmid, yeast artificial chromosome,
25 bacteriophage or eukaryotic viral DNA. Also, the host cell may be a bacterial cell (including gram positive cells), yeast cell, fungal cell, insect cell or animal cell. Suitable animals cells include, but are not limited to HeLa cells, Cos Cells, CV1 cells and various
30 primary mammalian cells. Culturing methods useful for permitting transformed or transfected host cells to produce polypeptides are well known in the art as are the methods for recovering polypeptides from such cells and for purifying them.

35

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Using the aforementioned method, this invention also provides a purified wildtype acylcoenzyme A: cholesterol acyltransferase II or III and a purified mutant acylcoenzyme A: cholesterol acyltransferase II or III.

5

This invention also provides an oligonucleotide of at least 15 nucleotides capable of specifically hybridizing with a unique sequence of nucleotides present within a nucleic acid which encodes a wildtype acylcoenzyme A: cholesterol acyltransferase II or III without hybridizing to a nucleic acid which encodes a mutant acylcoenzyme A: cholesterol acyltransferase II or III. Further, this invention also provides an oligonucleotide of at least 15 nucleotides capable of specifically hybridizing with a unique sequence of nucleotides present within the nucleic acid which encodes a mutant acylcoenzyme A: cholesterol acyltransferase II or III without hybridizing to a nucleic acid which encodes a wildtype acylcoenzyme A: cholesterol acyltransferase II or III. These oligonucleotide DNA or RNA. Such oligonucleotides may be used in accordance with well known standard methods for known purposes, for example, to detect the presence in a sample of DNA which will hybridize thereto.

25 The oligonucleotides include, but are not limited to, oligonucleotides that hybridize to mRNA encoding acylcoenzyme A: cholesterol acyltransferase II or III so as to prevent translation of the protein.

30 This invention also provides a nucleic acid having a sequence complementary to the sequence of the isolated nucleic acid which encodes acylcoenzyme A: cholesterol acyltransferase II or III.

35 This invention also provides a method for determining

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whether a subject known to have an imbalance in sterol levels has the imbalance due to a defect in esterification of sterol which comprises (a) obtaining from the subject an appropriate sample containing a mixture of all of the subject's nucleic acids; and (b) determining whether any nucleic acid in the sample from step (a) is, or is derived from, a nucleic acid which encodes a mutant acylcoenzyme A: cholesterol acyltransferase so as to thereby determine whether the subject's imbalance in sterol levels is due to a defect in esterification of sterol. The determination step (b) may comprises: (i) contacting the sample of step (a) with the isolated nucleic acid which encodes acylcoenzyme A: cholesterol acyltransferase II or III or the oligonucleotide of at least 15 nucleotides capable of specifically hybridizing with a unique sequence of nucleotides present within a nucleic acid which encodes a wildtype acylcoenzyme A: cholesterol acyltransferase II or III without hybridizing to a nucleic acid which encodes a mutant acylcoenzyme A: cholesterol acyltransferase II or III under conditions permitting binding of any nucleic acid in the sample which is, or is derived from, a nucleic acid which encodes a mutant acylcoenzyme A: cholesterol acyltransferase to the nucleic acid or oligonucleotide so as to form a complex; (ii) isolating the complex so formed; and (iii) identifying the nucleic acid in the isolated complex so as to thereby determine whether any nucleic acid in the sample contains a nucleic acid which is, or is derived from, a nucleic acid which encodes a mutant acylcoenzyme A: cholesterol acyltransferase II or III. In this method, both the isolation of any complex formed are effected using standard methods well known in the art.

In order to facilitate identification of the nucleic acid

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from step (a) the isolated nucleic acid or the oligonucleotide is labeled with a detectable marker. The detectable marker may be a radioactive isotope, a fluorophore or an enzyme. In additions, the nucleic acid sample may be bound to a solid matrix before performing step (I).

This invention also provides a method for treating a subject who has an imbalance in sterol levels due to a defect in esterification of sterol which comprises introducing an isolated nucleic acid which encodes a wildtype acylcoenzyme A: cholesterol acyltransferase II or III into the subject under conditions such that the nucleic acid expresses a wildtype acylcoenzyme A: cholesterol acyltransferase II or III, so as to thereby treat the subject.

This invention also provides a method for inhibiting wildtype acylcoenzyme A: cholesterol acyltransferase II or III in a subject which comprises transforming appropriate cells from the subject with a vector which expresses the nucleic acid complementary to the isolated nucleic acid which encodes a wildtype acylcoenzyme A: cholesterol acyltransferase II or III, and introducing the transformed cells into the subject so as to thereby inhibit wildtype acylcoenzyme A: cholesterol acyltransferase II or III. Further, in a preferred embodiment, the nucleic acid is capable of specifically hybridizing to a mRNA molecule encoding acylcoenzyme A: cholesterol acyltransferase II or III so as to prevent translation of the mRNA molecule.

This invention also provides a method for inhibiting the wildtype acylcoenzyme A: cholesterol acyltransferase II or III in a subject which comprises introducing an

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oligonucleotide of at least 15 nucleotides capable of specifically hybridizing with a unique sequence of nucleotides present within a nucleic acid which encodes a wildtype acylcoenzyme A: cholesterol acyltransferase II or III without hybridizing to a nucleic acid which encodes a mutant acylcoenzyme A: cholesterol acyltransferase II or III into the subject so as to thereby inhibit the wildtype acylcoenzyme A: cholesterol acyltransferase II or III. The oligonucleotide is capable of specifically hybridizing to a mRNA molecule encoding acylcoenzyme A: cholesterol acyltransferase II or III so as to prevent translation of the mRNA molecule.

This invention also provides for a method for identifying a chemical compound which is capable of inhibiting acylcoenzyme A: cholesterol acyltransferase II or III in a subject which comprises (a) contacting a wildtype acylcoenzyme A: cholesterol acyltransferase II or III with the chemical compound under conditions permitting binding between the acylcoenzyme and the chemical compound (b) detecting specific binding of the chemical compound to the acylcoenzyme; and (c) determining whether the chemical compound inhibits the activity of the coenzyme so as to identify a chemical compound which is capable of inhibiting acylcoenzyme A: cholesterol acyltransferase II or III in a subject.

This invention also provides method for differentially inhibiting one acylcoenzyme A: cholesterol acyltransferase but not others using the above methods. In an embodiment, only acylcoenzyme A: cholesterol acyltransferase I is inhibited. In another embodiment only acylcoenzyme A: cholesterol acyltransferase II (ARGP1) is inhibited. In an another embodiment only acylcoenzyme A: cholesterol acyltransferase III (ARGP2)

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is inhibited. Alternatively, two of the acylcoenzyme A: cholesterol acyltransferases may be inhibited. This invention further provides pharmaceutical compositions which will differentially inhibit one or more
5 acylcoenzyme A: cholesterol acyltransferases.

This invention also provides for a pharmaceutical composition comprising the chemical compound identified by the above-described method in an amount effective to
10 inhibit acylcoenzyme A: cholesterol acyltransferase II or III in a subject and a pharmaceutically effective carrier.

This invention also provides a method of treating a
15 subject who has atherosclerosis comprising the above-described pharmaceutical composition. A method of treating a subject who has hyperlipidemia comprising the above-described pharmaceutical composition.

This invention also provides a transgenic, nonhuman mammal comprising the isolated nucleic acid which encodes acylcoenzyme A: cholesterol acyltransferase II or III. The mammal includes, but is not limited to, a mouse, bovine, cat or dog.
20

This invention is illustrated in the Experimental Details section which follows. These sections are set forth to aid in an understanding of the invention but are not intended to, and should not be construed to, limit in any
25 way the invention as set forth in the claims which follow thereafter.
30

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Experimental Details

First Series of Experiments

Example 1:5 Materials and Methods:

Transformation of yeast was performed with lithium acetate (15) by amino-acid prototrophy selection. A diploid strain (5051) was constructed between two isogenic derivatives of W303 (16); W1346-3C (MATa, ade2-1, can1-100, his3-11, 15, leu2-3, 112, trp1-1, ura3-1) and W1134-2C (MATa, can1-100, his3-11, 15, leu2-3, 112, trp1-1, ura3-1, met14DHpaI-SalI). Growth on complete (YEPD) or synthetic medium, sporulation and dissection was performed as described (17).

Competent cells of *Escherichia coli* strain DH5a (Gibco-BRL) and DNA modifying enzymes (Promega) were used according to the manufacturers instructions. pH3(34), from L.A. Grivell, was digested with Nhe I, blunt-ended with Klenow sequences, and digested with Avr II to liberate a 1614-bp fragment. An Xba I, Sma I fragment of pJH-H1 encoding the *HIS3* gene was then inserted at these sites in the vector backbone to produce the *are1ΔNA* allele. This construct was digested with Bsa I to liberate a 3821-bp fragment which was then transformed into strain 5051. Disruption of *ARE1* was confirmed by Southern blot analysis.

30 Radioactive probes of acylcoenzyme A: cholesterol acyltransferase-related enzyme I were prepared by random priming (Pharmacia) with ³²P-dCTP. Genomic DNA (18) was transferred to Hybond membranes (Amersham) and hybridized in the absence of formamide at 65° or 60°C (19).

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A shotgun library of cosmid 14-21 from chromosome XIV (Peter Philippsen, Biozentrum Basel) was constructed using the nebulizing technique (20). The DNA was nebulized (90 seconds, 2 bars), size fractionated, treated with DNA polymerase I (Klenow fragment) and T4 DNA polymerase and blunt-end ligated into pTZ18R (Pharmazia, Germany). Nucleotide sequencing was performed by dideoxy-chain-termination with digoxigenin-labeled reverse primer and Sequenase (United States Biochemical). The reactions were analyzed on the GATC 1500 direct blotting electrophoresis system (GATC GmbH, Germany) using the Boehringer-Mannheim Dig-development protocol. Sequences were aligned by SeqMan (DNA Star Inc.). Database searching was performed with BLAST (21) and GCG Inc. software (22). The DNA sequence of the acylcoenzyme A: cholesterol acyltransferase-related enzyme I and acylcoenzyme A: cholesterol acyltransferase-related enzyme II genes are deposited at GenBank (P25628 and U51790, respectively).

K O - 5 a n d K O - 3 ' p r i m e r s
(GAGGGGACGAAAATTAGCCGCTATTAATTCTGGTATTGCCACCTAGACAAGAAG
TAAACAGACACAGATGcaagagttcgaatctcttagc (Sequence ID No.:
15) and CTATAAAGATTTAATAGCTCCACAGAACAGTTGCAGGATGCCTTAGGGT
CGActacgtcgtaaggccgtttctgac (Sequence ID No.: 16),
respectively; lower case corresponds to the *LEU2* gene)
were used in a PCR with the *LEU2* gene as a template to
produce the selectable yeast gene flanked by acylcoenzyme
A: cholesterol acyltransferase-related enzyme II gene
sequences (23). This was used to transform a derivative
of yeast strain 5051, heterozygous for the *are1ΔNA*
allele. To identify integrants at the acylcoenzyme A:
cholesterol acyltransferase-related enzyme II locus, a
PCR was performed on genomic DNA from these strains using

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are2-5' (CATTGCAGTTACACGTGAATGC) (Sequence ID No.: 17),
are2-3': (TAGCTCCACAGAACAGTTGCAGG) (Sequence ID No.: 18)
and a 3' ampimer corresponding to the *LEU2* gene (L2-3'
CTCTGACAACAACGAAGTCAG) (Sequence ID No.: 19).

5

1-2 units at an absorbance of 600nm of cells were
incubated in YEPD or defined media containing 1 μ Ci/ml
³H-oleate in tyloxapol/ethanol (1:1) for 16 hours. Total
10 lipids were prepared by hexane extraction (25) and
analysed by thin layer chromatography on DC-plastikfolien
kieselgel 60 plates (E-Merck, Germany). The plate was
developed in hexane, diethyl ether and acetic acid
(70:30:1) and stained with iodine vapor. Incorporation
15 of label into triglyceride and ergosterol ester was
ascertained following scintillation counting and
normalization to a ¹⁴C-cholesterol internal standard and
the dry weight of the cells.

20 To overexpress the acylcoenzyme A: cholesterol
acyltransferase-related enzyme I gene by copy number
under the control of its own promoter in YEp3-16, a 2354
bp Cla I fragment from pH3(34), encompassing the entire
acylcoenzyme A: cholesterol acyltransferase-related
25 enzyme I gene, was made blunt-ended with Klenow DNA
polymerase I and introduced into the Sma I site of
YEp352. To constitutively overexpress acylcoenzyme A:
cholesterol acyltransferase-related enzyme I from the ADH
promoter in pADH5-36, a 2290 bp Nar I fragment of
30 pH3(34), starting 70 bp 5' to the ORF was blunt-ended
with Klenow and ligated to Klenow-treated, Eco RI
digested, pDC-ADH (a derivative of pS5) (26). Increased
expression of the acylcoenzyme A: cholesterol
acyltransferase-related enzyme I transcripts, relative to
35 a wild-type cell, was confirmed by northern blot

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analysis.

The incorporation of [1-¹⁴C] acetate into saponified lipids was assessed as a measurement of sterol synthesis. Approximately 2 OD₆₀₀ units of cells were incubated with 20 μCi [1-¹⁴C] acetate in 2 ml defined media at 30°C for 3 hours and subjected to lipid saponification, hexane extraction and TLC chromatography (29). The incorporation of counts into total sterols were assessed following scintillation counting. To normalize the estimate of sterol biosynthesis to incorporation of acetate into the fatty acid pool, the aqueous lysate remaining after hexane extraction was acidified with concentrated HCl and re-extracted with hexane (30).

Experimental Discussion

To use yeast genetics to study sterol esterification, the human acylcoenzyme A: cholesterol acyltransferase sequence was used to search for homologous yeast genes and subsequently to identify an additional human isoform (Figures 1A and 1B). Acylcoenzyme A: cholesterol acyltransferase related enzyme I, an 1830-bp open reading frame (ORF) on yeast chromosome III, encodes a 610-residue protein with 23% identity and 49% similarity to human acylcoenzyme A: cholesterol acyltransferase I (Figures 1A and 1B). The yeast and human proteins possess leucine zipper motifs that could mediate protein-protein interactions (esterification is probably performed by a multimeric complex) (12), and possess at least two predicted transmembrane domains that may mediate the membrane association of the acylcoenzyme A: cholesterol acyltransferase reaction (13, 14).

To define the role of acylcoenzyme A: cholesterol

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acyltransferase-related enzyme I in sterol esterification, the deletion mutant, *are1 Δ NA*, was generated by homologous recombination (15, 16, 17) (Fig. 2A). In a diploid strain, a 1614-bp segment of one acylcoenzyme A: cholesterol acyltransferase-related enzyme I allele was replaced with the *HIS3* gene and confirmed by Southern hybridization (Fig. 2B). Analysis of mutant and wild-type haploid progeny from this diploid indicated no differences in growth rates or incorporation of ³H-oleate into ergosterol ester.

The lack of a defect in sterol esterification in *are1 Δ NA* strains could result from alternate esterification activities. Reduced stringency hybridization of yeast genomic DNA with the acylcoenzyme A: cholesterol acyltransferase-related enzyme I coding sequence as a probe indicated that additional homologous sequences were present (18, 19). A Bam HI digestion of genomic DNA produced the predicted 2.9-kb acylcoenzyme A: cholesterol acyltransferase-related enzyme I fragment and a ~6.0-kb hybridizing fragment (Fig. 2C). Contour clamped homogeneous electric field electrophoretic analysis of yeast chromosomes suggested the latter sequence was localized to chromosome X or XIV. On the basis of homology to acylcoenzyme A: cholesterol acyltransferase-related enzyme I, this gene, designated acylcoenzyme A: cholesterol acyltransferase-related enzyme II, encodes a second yeast homolog to human acylcoenzyme A: cholesterol acyltransferase I (Figures 1A and 1B). The genomic sequence (20, 21, 22) encompassing acylcoenzyme A: cholesterol acyltransferase-related enzyme II on chromosome XIV predicts a 5997-bp Bam HI fragment and a 1929-bp ORF, which translates into a 643-residue polypeptide. The yeast acylcoenzyme A: cholesterol

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acyltransferase related enzymes genes are 61% and 49% identical at the DNA and predicted protein levels, respectively. Arelp, Are2p and the human acylcoenzyme A: cholesterol acyltransferaseI protein are most related at the COOH-terminal region (42% identity over a 90-residue sequence) (Figures 1A and 1B).

To assess the contribution of Are2p to sterol esterification, one copy of the acylcoenzyme A: cholesterol acyltransferase-related enzyme II coding sequence was deleted from the genome of an *ARE1/are1ΔNA* heterozygous diploid by a polymerase chain reaction approach (23) (Fig. 2D). Haploid progeny representing the single *are1ΔNA* and *are2Δ* deletions and the *are1ΔNAare2Δ* double mutant were obtained. To ascertain the effect of deletion of acylcoenzyme A: cholesterol acyltransferase-related enzymes genes upon cytoplasmic lipid storage, the neutral lipid components (triglyceride and sterol ester) of the yeast cells were detected by fluorescence microscopy after staining with Nile Red (24). In wild-type cells, cytoplasmic fluorescent droplets accumulated in stationary phase cultures (Fig. 3A). No differences in are single mutants were detected. However, the number of droplets observed in *are1ΔNAare2Δ* double mutants, was one-third to that in wild-type strains (Fig. 3B; over multiple fields, 5.57 ± 2.73 vs. 16.73 ± 4.6 droplets/cell, $P < 0.05$).

The wild-type and are mutant cells were analyzed for the incorporation of ^3H -oleate into sterol ester (25) (Fig. 4B). No significant differences in triglyceride biosynthesis were detected. In contrast to normal sterol ester biosynthesis observed in *are1ΔNA* mutants, deficiencies in sterol esterification were apparent in

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both *are2Δ* and *are1ΔNAare2Δ* mutants. These were detected by iodine vapor staining of thin layer chromatographs of total yeast lipids in addition to the oleate incorporation assays. Sterol ester levels of *are2Δ* single mutants were reduced to less than 26% of wild-type suggesting the acylcoenzyme A: cholesterol acyltransferase-related enzyme II isoform to confer the majority of acyltransferase activity. The *are1ΔNAare2Δ* double mutant was almost totally deficient in sterol esterification (less than 1% of wild-type levels). In confirmation of the critical role of Are proteins in sterol esterification, microsomes from double mutant yeast cells lacked acylcoenzyme A: cholesterol acyltransferase activity when assayed *in vitro*.

To confirm that the protein encoded by an acylcoenzyme A: cholesterol acyltransferase-related enzymes ORF was sufficient for sterol esterification, the acylcoenzyme A: cholesterol acyltransferase-related enzyme I coding sequence was over-expressed in vectors with increased copy number (YE_p3-16) or elevated transcription (the alcohol dehydrogenase promoter in pADH5-36) (26). There were no detectable changes in triglyceride or phospholipid biosynthesis resulting from acylcoenzyme A: cholesterol acyltransferase-related enzyme I over-expression. In *are2Δ* or *are1ΔNAare2Δ* double mutants, acylcoenzyme A: cholesterol acyltransferase-related enzyme I over-expression complemented the sterol esterification defect (Fig. 4C). In wild-type and *are1ΔNA* single mutants, the high level expression of acylcoenzyme A: cholesterol acyltransferase-related enzyme I did not elevate sterol ester synthesis above untransformed controls. This suggests that either substrates are limiting in acylcoenzyme A: cholesterol

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acyltransferase-related enzymes strains or that the enzyme is post-translationally regulated as in mammalian cells (27).

5 An accumulation of unesterified sterol in cell membranes would likely be deleterious (28). However, despite the major changes in sterol esterification conferred by the are mutants, we did not detect any reduction in growth rates. The established role of sterol esterification in
10 the storage of sterol suggests that an inability to esterify sterol could lead to homeostatic changes in sterol biosynthesis. This relationship might account for the viability of the mutants. Total lipids, labelled by the incorporation of [1-¹⁴C] acetate into exponentially
15 growing cells (29, 30), were saponified and extracted. The *are1 ΔNaare2Δ* double mutants had a two to three-fold lower level of sterol biosynthesis than wild-type cells, although no changes were observed in the single mutants (Fig. 4D). In fact, free sterol concentrations were
20 roughly equivalent in all cells. Feedback regulation of sterol biosynthesis by acylcoenzyme A: cholesterol acyltransferase activity has been observed in mammalian cells (31) and may be a common mechanism that maintains intracellular sterol at non-toxic concentrations.

25 The involvement of multiple gene families in sterol homeostasis is common in mammalian and yeast cells, for example, the LDL receptor related protein and scavenger receptor gene families, the SREBP family, and 3-hydroxy-
30 3-methyl-glutanyl-CoA reductase) (4, 32, 33, 34). This apparent redundancy of function has clear physiological consequences as evidenced by deletion of any one of the family members. The observation here of two yeast genes for sterol esterification provoked the hypothesis of

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similar redundancy for this reaction in humans. To this end, a consensus of the yeast acylcoenzyme A: cholesterol acyltransferase-related enzymes and human acylcoenzyme A: cholesterol acyltransferaseI sequences was used to
5 identify an additional cDNA with significant identity (47%) to human acylcoenzyme A: cholesterol acyltransferaseI and the yeast proteins (Figure 1B, Genbank accession # R07932).

10 Sterol homeostasis is a complex event under subtle regulatory controls, one component of which is sterol esterification. The demonstration here of multiple yeast and human acylcoenzyme A: cholesterol acyltransferase isoforms raises the possibility that *in vivo*, the enzymes
15 exhibit alternate substrate preferences. The analysis of esterification reactions in yeast is likely to impact the understanding of sterol homeostasis and atherosclerosis in humans.

20 **Example 2:**

Tissue specific expression of acylcoenzyme A: cholesterol acyltransferase II was analyzed by Northern blot RNA hybridization of RNA obtained from the described tissues.
25 Using the same materials and procedures of Chang, et al. (10), the specific expression of acylcoenzyme A: cholesterol acyltransferase II in liver and muscle is documents, in contrast to similar experiments using the previously known acylcoenzyme A: cholesterol
30 acyltransferase I (10) (Figures 7A and 7B). Acylcoenzyme A: cholesterol acyltransferase II was also detected and specifically expressed in adrenal, thyroid and testicular tissues.

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Example 3:

After determining the consensus sequence between the two yeast gene and the previously known human acylcoenzyme A: cholesterol acyltransferase, the consensus sequence was compared to sequences deposited in Genbank. The clones containing the sequences that showed similarity to the consensus sequence were ordered from the I.M.A.G.E. Consortium, affiliated with Research Genetics, Inc., 2130 Memorial Parkway S.W. Huntsville, Alabama 35801. Clones deposited with the I.M.A.G.E. consortium are publicly available upon request. A particular clone, Genbank ID clone No. Z39933 was chosen. This clone contains a cDNA fragment whose sequence encodes human acylcoenzyme A: cholesterol acyltransferase II. The fragment was cut out with restriction enzymes Bgl II and Not I. The resulting fragment was introduced into the yeast expression vector pRS426 at Bgl II and Not I sites downstream of the yeast promoter (GAL1/GAL10) which is regulated by carbon sources. The resultant vector was designated YepAB-ACAT2 (Figure 6).

Example 4:

Antisense RNA technology can be used to create mice, or mouse or human cell lines incapable of translating acylcoenzyme A: cholesterol acyltransferase II RNA into protein. Standard methods may be used to create an antisense oligonucleotide to the human homolog of acylcoenzyme A: cholesterol acyltransferase II. These methods are well known in the art (36).

Specifically, part or all of a wildtype acylcoenzyme A: cholesterol acyltransferase II is ligated adjacent to a mammalian promoter in the opposite orientation. The

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promoter and other replicatory mechanisms inside the cell will transcribe a human homolog of acylcoenzyme A: cholesterol acyltransferase II encoding, nonsense strand. This strand will bind with the coding mRNA which is normally synthesized to form a complex. Due to the formation of this complex, the antisense strand prevents the translation of the coding mRNA into protein.

Further, one skilled in the art can synthesize an oligonucleotide *in vitro* which is capable of binding the mRNA that encodes a human homolog of acylcoenzyme A: cholesterol acyltransferase II so as to inhibit the translation of the mRNA into protein. The oligonucleotides can then be introduced into the subject using a pharmaceutically acceptable carrier. Methods of synthesizing naturally and non-naturally occurring oligonucleotides which are capable of inhibiting the translation of the mRNA into protein are well known in the art. Also, means of transfecting an organism with such oligonucleotides are well known in the field.

Example 5:

Mice can be made with an alteration in their genome, specifically at the acylcoenzyme A: cholesterol acyltransferase II gene site. Standard methods may be used to alter the genome. These methods are well known in the art (37, 38).

One such process to achieve this goal involves disrupting the wildtype mouse homolog of acylcoenzyme A: cholesterol acyltransferase II *in vitro*, then introducing the altered gene into mouse embryonal stem cells in such a way as to target integration into the corresponding genomic region. This process can be performed such that both copies of

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- the wildtype acylcoenzyme A: cholesterol acyltransferase II are replaced by the altered, knock-out version. These modified cells can be introduced into blastocysts which will be allowed to develop into chimeric adults. Mice
- 5 bearing the altered acylcoenzyme A: cholesterol acyltransferase II gene will be mated to each other to generate homozygous mutant acylcoenzyme A: cholesterol acyltransferase II animals.
- 10 Further, one can breed two mice who are heterozygous for mutant acylcoenzyme A: cholesterol acyltransferase II. From their progeny, one skilled in the art could select the progeny who are homozygous for mutant acylcoenzyme A: cholesterol acyltransferase II. Breeding and selecting
- 15 such progeny are well known in the art.

References of the First Series of Experiments

1. T.-Y. Chang and G.M. Doolittle, in *The Enzymes*, P. Boyer, Eds. (Academic Press, New York, 1983), vol. XVI, pg. 523.
- 5 2. K. E. Bloch, *CRC Crit. Rev. Biochem.* **14**, 47 (1983).
3. M. P. Reinhart, J. T. Billheimer, J. R. Faust, J. L. Gaylor, *J. Biol. Chem.* **262**, 9649 (1987).
4. X. Wang et al., *Cell* **77**, 53 (1994).
5. J. T. Billheimer and P. J. Gillies, in *Advances in Cholesterol Research* M. Esfahani, J. B. Swaney, Eds. (Telford Press, Philadelphia, 1992).
- 10 6. T. P. Carr, J. S. Parks, L. L. Rudel, *Arterio. and Thromb.* **12**, 1275 (1992).
7. S. C. Rumsey, N. F. Galeano, B. Lipschitz, R. J. Deckelbaum, *J. Biol. Chem.* **270**, 10008 (1995).
- 15 8. T. Shiratori and D. S. Goodman, *Biochem. Biophys. Acta* **106**, 625 (1965).
9. S. S. Katz, G. G. Shipley, D. M. Small, *J. Clin. Inv.* **58**, 200 (1976).
- 20 10. C. C. Y. Chang, H. Y. Huh, K. M. Cadigan, T. Y. Chang, *J. Biol. Chem.* **268**, 20747 (1993).
11. D. Cheng, C. C. Y. Chang, X. M. Qu, T. Y. Chang, *J. Biol. Chem.* **270**, 685 (1994).
12. S. K. Erickson, S. R. Lear, M. J. McCreery, *J. Lipid Res.* **35**, 763 (1994).
- 25 13. D. S. Goodman, D. Deykin and T. Shiratori, *J. Biol. Chem.* **239**, 1335 (1964).
14. G. M. Doolittle and T. Y. Chang, *Biochem.* **21**, 674 (1982).
- 30 15. H. Ito, Y. Fukuda, K. Murata, A. Kimura, *J. Bacteriol.* **153**, 163 (1983).
16. B. J. Thomas and R. Rothstein, *Cell* **56**, 619 (1989).
17. F. Sherman and J. Hicks, in *Methods in Enzymology* C. Guthrie, G. R. Fink, Eds. (Academic Press, 1991),

- vol. 194, pp. 21.
18. C. S. Hoffman and F. Winston, *Gene* **57**, 267 (1987).
 19. F. M. Ausubel et al., *Current Protocols in Molecular Biology* (John Wiley & Sons, New York, 1987), vol. 1.
 - 5 20. C. M. Okpodu et al., *BioTechniques* **16**, 154 (1994).
 21. S. F. Altschul et al., *J. Mol. Biol.* **215**, 403 (1990).
 22. J. Devereux, P. Haeberli, O. Smithies, *Nucl Acids Res* **12**, 387 (1984)
 - 10 23. A. Baudin, O. Ozier-Kalogeropoulos, A. Denouel, C. Cullin, *Nuc. Acids Res.* **21**, 3329 (1993).
 24. P. Greenspan, E. P. Mayer, S. D. Fowler, *J. Cell Biol.* **100**, 965 (1985).
 25. L. W. Parks, C. D. Bottema, R. J. Rodriguez, T. A. Lewis, *Methods Enzymol* **111**, 333 (1985).
 - 15 26. S. L. Sturley et al., *J. Biol. Chem.* **269**, 21670 (1994).
 27. C. C. Chang et al., *J. Biol. Chem.* **270**, 29532 (1995).
 - 20 28. G. J. Warner et al., *J. Biol. Chem.* **270**, 5772 (1995).
 29. R. Y. Hampton and J. Rine, *J. Cell Biol.* **125**, 299 (1994).
 30. D. Dimster-Denk, M. K. Thorsness, J. Rhine, *Mol. Biol. Cell* **5**, 655 (1994).
 - 25 31. I. Tabas, D. A. Weiland, A. R. Tall, *J. Biol. Chem.* **261**, 3147 (1986).
 32. M. Krieger and J. Herz, *Annu. Rev. Biochem* **63**, 601 (1994).
 - 30 33. X. Wang et al., *Cell* **77**, 53 (1994).
 34. M. E. Basson, M. Thorsness, J. Rine, *Proc. Natl. Acad. Sci. USA* **83**, 5563 (1986).

-41-

35. S. L. Thompson, R. Burrows, R. J. Laub, S. K. Krisans, *J. Biol. Chem.* **262**, 17420 (1987).
36. Griffin, E.F. and H. Harris (1992) *J. Cell Science* **102**: 799-805.
- 5 37. Joyner, A.L. and F. Guillemot (1994) *Current Opinion in Neurobiology* **4**: 37-42.
38. Duyao, M.P., et al. (1995) *Science* **269**: 407-410.

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5 cond Series of Experiments

The efficient regulation of intracellular sterol levels is required for cell viability by all eukaryotic organisms. When this regulation is aberrant in cells of the arterial wall, disease states such as atherosclerosis ensue. A critical component of this homeostasis is intracellular sterol esterification reaction, mediated by the enzyme, acyl coenzyme A-cholesterol acyltransferase (ACAT). In the model eukaryote, yeast, this laboratory has demonstrated that sterol esterification is mediated by a two gene family (Yang et al., Science, 1996, 272:1353). The existence in human cells of two additional genes encoding ACAT related enzymes are demonstrated. These protein are termed ACAT related gene products (ARGP) 1 and 2, also known as acylcoenzyme A: cholesterol acyltransferase II and acylcoenzyme A: cholesterol acyltransferase III respectively. The ARGPs exhibit marked sequence conservation to the human ACAT sequence (hACAT) originally identified by Chang and colleagues. ARGP1 is expressed at high levels in intestine and liver in contrast to the expression of hACAT which is of low abundance in these tissues. The observation that knock-out mutant mice deficient in the murine homolog if hACAT retain sterol esterification activity in liver and intestine (Meiner et al., PNAS, 1996, 93:14041), suggests that ARGP1 is a candidate for sterol esterification in these tissues. The expression of ARGP2, by contrast, seems to be restricted to the fetal liver, suggesting it to have a role in lipid metabolism during development. Analysis of genome databases indicates that ACAT-like gene families are a common occurrence in multiple organisms. It is hypothesize that multiple enzymes for sterol esterification will provide flexibility in response to differing sterol and fatty acid substrates encountered by different tissues. This further suggests specific roles

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for these enzymes in lipoprotein production, lipid homeostasis, and disease progression.

5 The regulation of membrane sterol levels is required for cell viability by all eukaryotic organisms. When this regulation is aberrant in human cells, disease states such as atherosclerosis (excessive accumulation of cellular esterified cholesterol in cells of the arterial wall, reviewed in (1-4)), Niemann Pick C (inability to store sterol correctly, resulting in lysosomal lipidosis, (5)) or Wollmann's disease (a defect in sterol ester hydrolysis, (6)) ensue. A critical component of this homeostasis is the intracellular neutralization of sterol by an esterification reaction between the C₃-OH group of cholesterol and fatty acyl-coenzyme A. This reaction is performed in mammalian cells by the enzyme acyl coenzyme A-cholesterol acyltransferase (ACAT). Since the process of sterol esterification converts sterol into a cytoplasmic storage form, it is critical to all eukaryote, including the microorganism Saccharomyces cerevisiae (budding yeast). Analysis of sterol homeostasis in this model organism has the advantage that molecular genetics, particularly since the completion of the yeast genome sequencing project, is powerful and relatively straightforward. Taking advantage of this, it is demonstrated that sterol esterification in yeast is mediated by a two gene family (7), neither of which is essential for life. These genes (ARE1 and ARE2; encoding ACAT Related Enzymes 1 and 2, respectively) are both capable of independently esterifying sterol, although in terms of contribution to the sterol ester mass of the cell, Are1 is a minor isoform relative to Are2. The genes are structurally and functionally analogous to the ACAT sequence isolated originally from macrophages by Chang and colleagues (8). They share approximately 23% identity at the protein level and expression of the human macrophage ACAT cDNA in yeast are

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double deletion mutants results in esterification of sterol (9).

5 A critical test of the role of the ACAT gene product in cholesterol homeostasis and atherosclerosis was initiated by Farese and colleagues, by the production of "knock-outs" at the Acact locus corresponding to the mouse homolog of hACAT (10). The fidelity of the mutation was confirmed by sequencing of cDNA from the disrupted allele and by the failure to detect immunoreactive protein in Acact-/- cell extracts. The animals were healthy and fertile and had residual, but significant, sterol esterification activity in fibroblasts and macrophages. Cholesterol ester levels and ACAT activity in the adrenals were also severely reduced. Conversely, Acact-/- 15 livers contained significant levels of cholesterol ester, and esterification activity was not altered. Furthermore, sterol absorption in the intestine, a process that probably requires esterification, was unaffected by the gene disruption. These observations 20 strongly suggest that as in yeast, there are multiple genes for the ACAT reaction in mammalian cells, probably with tissue specific expression patterns.

25 Interestingly, despite the clear origin of the yeast gene family by gene duplication, the ARE proteins have diverged such that the majority of sequence conservation is in the COOH-terminal domain of the protein. This is presumably the critical region of the molecule, since it 30 is also conserved in the human protein. Using this region as a database probe, R07932 (11) was identified, a partially sequenced cDNA entry in the database of expressed sequence tags (best); R07932 exhibits significant similarity to the ACATs particularly over the COOH-terminal region. Taken together; the "founder" 35 sequence, the observations in yeast of a two gene family for sterol esterification, and the tissue-specific

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expression patterns of enzyme activity in Acact-knock-out mice, suggest that there are multiple genes for this reaction in all eukaryote. It is reported here the isolation and characterization of cDNAs from two human loci that encode ACAT Related Gene Products (ARGP). ARGP1 is represented multiple times in the best, including R07932, and is expressed ubiquitously with the highest levels occurring in the liver, intestine and adrenal gland. By contrast, sequences identical to ARGP2 in the databases are infrequent, consistent with the observation of an essentially embryonic pattern of expression. Analysis of genome databases indicates that gene families that conserve these motifs are a common occurrence in multiple organism.

15

Materials and Methods.

Database searching for ACAT related sequences. A sequence corresponding to the strongest region of protein conservation between the human macrophage ACAT and yeast ARE sequences was used to identify protein sequences predicted to be encoded by entries in the best using the tblastn software (NCBLI). The DNA sequences thus arising were used to detect additional clones in any available database, that demonstrated overlaps of nucleotide sequence identity. Databases searched included; best, the non-redundant GENBANK, and the confidential database held at The Institute of Genome Research (TIGR). Overlaps between these sequences were detected using the sequence alignment programs, "lineup" and "pileup" from GCG Inc (Madison, WI). A consensus sequence was then generated. Escherichia coli clones with the largest inserts corresponding to these sequences (see table 1) were obtained from the I.M.A.G.E. consortium and resequenced from both ends using commercial primers, T3 and T7, or internal primers derived from a consensus. Nucleotide sequencing was performed at the Columbia University Combined Center core facility using an Applied

35

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Biosystems fluorescent sequencing machine.

Table 1: Entries of human ACAT related gene products in the products in the data base of expressed sequence tags.

Gene	Clone ID	GENEBANK ID	Insert size (bp)	Comments
ARGP1	200587	R99213	620	
		R99214		
	55218	c-IMF11	1800	chimera
		Z43867		
		Z33993		
	1881180	H45923	1000	
		H45924		
	78614	M79086	300	
	153836	R48474	800	
		R48475		
ARGP2	106260	T35085	800	
	128921	R10272	680	
		R10273		
	213176	N75438	540	
	245265	H76642	300	

Isolation and sequencing analysis of full length cDNA clones of ARGP1 and ARGP2. Since in no instance were any of the database clones full length for either ARGP1 or ARGP2, additional clones with intact 5'-ends are described. Several strategies were chosen using a consensus nucleotide sequence derived from the sequencing of the best clones designed and synthesized 3-end, gene specific primers and used a PCR based, rapid amplification of cDNA ends (RACE) to derive 5'-RACE reaction products from a human liver/spleen Marathon library (Clontech®). Similar strategy was used to derive PCR products from a human fetal brain library generously

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provided by Bento Soares (Columbia University). In some instances, a nested PCT reaction was performed using internal gene specific primers and library adaptors. Finally primer extension cDNA products were identified from mRNA extracted from human intestine (a kind gift of P. Dawson). Amplification products of the predicted size were confirmed as gene specific, using southern hybridization to sequences predicted to be at the 3'-end of these products. The products were isolated from agarose gels using Geneclean and subcloned into TA variants of pBluescript (Stratagene®) vectors of klenow/kinase treated and blunt end ligated to pGEM2 (Promega®). Positive clones were identified by colony hybridization or by PCR amplifications using an internal ARGP specific primer. Clones with the largest inserts were sequenced to obtain novel sequence and where necessary, this process was reiterated with ARGP 5' specific primers derived from the new sequence.

Tissue specific expression of hACAT and ARGps. Fragments of the best clones R99213 and R10273 corresponding to ARGP1 and ARGP2, respectively were derived by digestion with EcoRI and NotI, and purified from agarose gels with Geneclean. A 1.6 kbp fragment corresponding to the human ACAT cDNA identified by Chang et al was used as a probe for the expression of this gene. Radiolabelled probes were generated by random priming (Pharmacia®) in the presence of ³²-P dCTP and used to probe Multiple Tissue Northern (MTN, Clontech®) of human samples. Hybridizations were performed, according to the manufacturers instructions, using ExpressHyb rapid hybridization solution for 1 hour at 78°C, followed by washed in 2xSSC at 55°C and 0.1xSSC, 0.5%SDS at 50°C.

Cell culture expression of ARGPs. To facilitate quantitation of mRNA from the ARGP genes, a reverse-transcriptase PCR (RT-PCR) approach was devised to

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analyze expression in a variety of human (HeoG2, THP-1 macrophages) and rodent (J774 macrophages) and simian (CV1 kidney cells). Where possible, primers were designed to be conserved between rodents and humans (as described below, the mouse sequence homolog to ARGP1) has been identified. Alternatively, PCR conditions were optimized to permit moderate mismatches. The ARGP amplification primers were designed to be gene specific (i.e. to regions not conserve within the family) and to produce distinct size products.

Experimental Results and Discussion

The approach that the region of strongest conservation between the yeast ARE proteins and hACAT would be critical to the function of any sterol esterification enzymes was taken. A region of conservation (consensus; LN---E---FGDR-FY GDWWN, single letter amino-acid code) that is invariant over the three proteins was chosen and a series of entries derived from gene sequencing projects identified. In addition to sequences from Caenorhabditis. elegans, Schizosacharomuces pombe, Drosophila melanogater and Arabidopsis thaliens, several entries in the best of human cDNAs that suggested an independent gene encoding an ACAT like protein were observed. Using the nucleotide sequence to this clone, a second homologous but distinct entry was identified. These proteins are termed, ACAT Related Gene Products (ARGP) 1 (acylcoenzyme A: cholesterol acyltransferase II) and 2 (acylcoenzyme A: cholesterol acyltransferase III). The sequence identified by Chang et al (8) will be referred to as hACAT, hereon. A limited protein sequence to a founder clone (R07932) to ARGP1 has been presented previously (11). The entries in the best that define these two genes, including their insert sizes are described in table 1. As is evident, the majority of inserts (with the exception of a chimeric clone ZA3867) are less than 1Kbp. The northern and sequence analysis

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presented indicated them to be incomplete clones. However, they clearly define two distinct genes of strong similarity to the ACAT sequence, with the majority of predicted protein conservation at the COOH-terminal region. As described below certain motifs considered critical to sterol esterification are conserved. To identify the role of these genes in the reaction, full length ARGP clones were sought and their patterns identified.

ARGP1, a ubiquitously expressed member of the ACAT gene family. To establish the profile of expression of ARGP1, probed multiple tissue northern of human mRNA was probed, using a fragment close to the 3' end of the gene. Although this region displays the maximum conservation at the protein level in this gene family, the genes are sufficiently divergent at the DNA level to be able to design gene specific hybridization probes. The ARGP1 sequence is expressed at abundant levels in may tissues with the exception of lung and kidney. The majority of tissues express a 2.0kb message but, some tissues (e.g. adrenal, small intestine, thymus) also express a 2.4kb mRNA at varying levels. The same northern were hybridized with a probe to the human macrophage ACAT sequence. As described by others(8,12,13), the hACAT sequence detects 4 messages of approximately 3.0, 4.0, 4.7 and 7.4Kb. Upon comparison of the two hybridization results, an overlapping but occasionally differential expression pattern was observed. Adrenal tissues express the highest levels of both hACAT and ARGP1 message. By this analysis, hACAT messages are rare in liver and intestine in contrast to ARGP1 which is highly expressed in these tissues. Conversely, ARGP1 was poorly expressed in kidney, lung and placenta although hACAT mRNA was easily detected. This tissue specific expression suggests that ARGP1 is an ideal candidate for sterol esterification in tissues such as liver and intestine,

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which retain sterol esterification activity in ACAT k/o mice (10).

ARGP2, an embryonic isoform of the ACAT gene family.

5 Efforts to identify a transcript from ARGP2 in adult tissues were unsuccessful. Therefore embryonic tissue samples were chosen to investigate since the original founder clone was derived from a fetal liver library. A multiple tissue northern of mRNA from human embryonic
10 brain, liver, kidney, and lung, were probed with and ARGP2 specific, COOH-terminal probe. As shown in figure 9, a single message of ~2.2kb was identified only in embryonic liver tissues, suggesting a high degree of tissue and developmental specificity to the expression of
15 this gene product.

Expression of ARGP1 in cell culture models. To develop a system in which to test the effect of reaction substrates on the esterification reaction performed by
20 the ARGP enzymes. The expression of these genes in several tissue specific were examined, cell culture models. As shown in figure 10, ARGP1 is clearly expressed in liver (HepG2) and Kidney (CV-1) cell lines. The latter result is somewhat in contrast to the northern
25 blot on human tissue samples. This most likely reflects the sensitivity of the RT-PCR approach compared to filter hybridization and suggests that ARGP1 is probably expressed in most tissues. Alternatively it may represent species difference (simian vs. human) or more
30 interestingly the differentiation status of the cells under study. In data not shown here, ARGP1 was also clearly expressed in human and mouse macrophage models (THP-1 and J774 cells).

35 Sequence characteristics of ARGP1 and ARGP2. By a combination of 5'-RACE and primer extension additional sequence to cDNA s for ARGP1 and ARGP2 (Figs. 11 and 12)

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have been identified. The ARGP1 sequence predicts a 407 amino-acid protein with approximately 27% identity and 52% similarity to the hACAT protein (Fig. 4). Interestingly, as it was observed for the yeast ARE proteins, the strongest conservation exists at the COOH-terminus of the molecules, to the extent that the NH-2-terminal 50% of all these proteins is essentially unrelated sequence. This pattern also persists at the DNA level (not shown). Identification of the genomic sequence to these cDNAs will establish whether this remarkable divergence arises by exon shuffling of common sequences. Alternatively, convergent evolution of domains with conserved functions in sterol esterification or related processes, may have resulted in the generation of these families. Since the level of DNA conservation between ARGP1 and hACAT is quite low (37% identity), the latter possibility seems likely. The conserved regions are discussed in the context of multiple ACAT like sequences below. The ARGP1 sequence predicts a protein of approximately 47kDa with multiple transmembrane domains in similar positions to those predicted in hACAT. This strongly suggests a membrane location for ARGP1 as would be predicted for a sterol esterification enzyme.

ARGP2 displays a significantly higher level of amino acid conservation with hACAT than does ARGP1. Over the sequence shown (Fig. 12), the protein is 59% identical and 79% similar to human ACAT. Over the same region ARGP1 is only conserved at the level of 32% identity. This striking identity is maintained at the DNA level (62% identity) and may suggest that ARGP2 is more closely analogous to hACAT in both its mechanism of action and its origin, than is ARGP1. As for ARGP1, certain hallmark sequences are retained in ARGP2 (see below). The ARGP2 predicted protein also possesses several predicted transmembrane domains. One entry to the best for ARGP2 has also been allocated an STS (sequence tagged

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site) at the Whitehead Institute, (entry # WI-11660) and has thus been mapped to human chromosome 12.

5 Sterol esterification enzymes evolve as gene families in
multiple organisms. Using the hACAT and AGRP nucleotide
sequences as probes of multiple databases, we sought to
establish whether the observation of gene families of
ACAT related enzymes in yeast and humans was a common
10 occurrence in other organisms. In general this is the
case (Fig. 13). Sequences from the genome of C. elegans,
D. melanogaster and S. pombe, have been identified that
are distinct from each other, within an organism, and
exhibit approximately 25% identity at the predicted
protein level. As for all the ACAT-like proteins, the
15 maximum conservation is observed at the COOH-terminal
region, with many of the apparently critical motifs
described below, being maintained. As would be
anticipated the mouse cDNA for ARGP1 exhibits
approximately 85% identity with its human homolog.

20 Sequence conservation between ARGPs and ACAT in multiple
organisms. As described above, these sequences are
ubiquitous. This conservation, across and within
organisms, facilitates the identification of critical
25 domains of esterification enzymes (Fig. 14).
Interestingly, there is no sequence similarity between
any ACAT-like molecule and lecithin cholesterol
acyltransferase (LCAT), despite the shared utilization of
cholesterol. For the hACAT sequence and its murine
30 homologs, a similarity to "signature" motifs of enzymes
involved in acyl adenylation reactions was reported (8,
12). However, these sequences are unlikely to be
critical, since they are not conserved in any homolog
from any other organism. By contrast, there are regions
35 of strong conservation between these molecules which may
be critical to function. In the esterification
defective, SRD4 mutant CHO cell line, the expressed but

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defective ACAT allele encodes a single amino-acid substitution of leucine₂₆₅ lies in a conserved domain of human, rodent and yeast ACAT. Interestingly, this motif in ARGP1 is more degenerate, although the serine is conserved, the flanking sequence is conservatively replaced by similar residues. The ACAT reaction is probably mediated by a multimeric complex, as shown by radiation inactivation experiments (15). Accordingly, the yeast and human sequences all possess "leucine zipper" multimerization motifs. ARGP1 and ARGP2 lack a classical multimerization motif. Although protein phosphorylation as a mode of ACAT regulation has been refuted (16), a very strong region of conservation (consensus over 7 sequences; LN---E---FGDR-FYGDWWN, single letter amino-acid code) predicts a tyrosine kinase consensus motif for phosphorylation. ARGP2 and ARGP1 are no exception to this. In particular the aspartic acid-tryptophan-tryptophan-asparagine (DWWN) sequence appears to be invariant (with the exception of S.pombe, where it is AWWN) and may represent an active site for the esterification reaction. These regions of conservation are targets for mutagenesis and in preliminary experiments appear critical to the activity of the ACAT and ARE enzymes (17).

Why ACAT gene families? The role, if any, of these ACAT sequence homologs in sterol homeostasis is unclear. Since mouse macrophage ACAT is not critical to sterol esterification in the liver and intestine, it is possible that the additional enzymes evolved to recognize alternate substrates and thus promote sterol absorption in the intestine or production of lipoproteins by the liver (39). Future experiments will be directed to complete the molecular characterization of these genes and test these hypotheses.

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References of the Second Series of Experiments

1. Liscum, L., and Underwood, K.W. (1995) J. Biol. Chem. **270**(26), 15443-15446
2. Chang, T.Y., Chang, C.C.Y., and Cadigan, K.M. (1994) Trends Cardiovascul. Med. **4**, 223-230
3. Tabas, I. (1995) Curr. Op. in Lipidol. **6**(5), 260-268
4. Libby, P., and Clinton, S.K. (1993) Curr. Opin. Lipidol. **4**, 3355-363
5. Pentchev, P.G., Brady, R.O., Blanchette-Mackie, E.J., Vanier, M.T., Carstea, E.D., Parker, C.C., Goldin, E., and Roff, C.F. (1994) Biochim. Biophys. Acta **1225**, 235-243
6. Goldstein, J.L., Dana, S.E., Faust, J.R., Beaudet, A.L., and Brown, M.S. (1975) Journal of Biological Chemistry **250**(21), 8487-95
7. Yang, H., Bard, M., Bruner, D.A., Gleeson, A., Deckelbaum, R.J., Aljinovic, G., Pohl, T., Rothstein, R., and Sturley, S.L. (1995) Circulation In Press
8. Chang, C.C.Y., Huh, H.Y., Cadigan, K.M., and Chang, T.Y. (1993) J. Biol. Chem. **268**(28), 20747-20755
9. Yang, H., Cromley, D., Wang, H., Billheimer, J.T., and Sturley, S.L. (1997) J. Biol. Chem. **272**, 3980-3985
10. Meiner, V.M., Cases, S., Myers, H., Snade, E.R., Bellosta, S., Schambelan, M., Pitas, R.E., McGuire, J., Herz, J., and Farese, R.V. (1996) Proc. Natl. Acad. Sci. USA **93**, 14041-14046
11. Yang, H., Bard, M., Bruner, D.A., Gleeson, A., Deckelbaum, R.J., Aljinovic, G., Pohl, T., Rothstein, R., and Sturley, S.L. (1996) Science **272**, 1353-1356
12. Uelman, P.J., Oka, K., Sullivan, M., Chang, C.C.Y., Chang, T.Y., and Chan, L. (1995) J. Biol. Chem. **270**, 26192-26201
13. Pape, M.E., Schultz, P.A., Rea, T.J., DeMattos, R.B., Kieft, K., Bisgaier, C.L., Newton, R.S., and

-55-

- Krause, B.R. (1995) Journal of Lipid Research 36(4), 823-38
14. Cao, G., Goldstein, J.L., and Brown, M.S. (1996) Journal of Biological Chemistry 271(24), 14642-8
- 5 15. Erickson, S.K., Lear, S.R., and McCreery, M.J. (1994) J. Lipid Res. 35, 763-769
16. Corton, J.M., and Hardie, D.G. (1992) European Journal of Biochemistry 204(1), 203-8
- 10 17. Guo, Z., Yang, H., and Sturley, S.L. (1996) Circulation 94, I-35

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (I) APPLICANT: Stephen L. Sturley
- (ii) TITLE OF INVENTION: DNA ENCODING ACYLCOENZYME A: CHOLESTEROL
ACYLTRANSFERASE 11 AND USES THEREOF
- (iii) NUMBER OF SEQUENCES: 19
- (iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE:
(B) STREET: 1185 Avenue of the Americas
© CITY: New York
(D) STATE: New York
(E) COUNTRY: U.S.A.
(F) ZIP: 10036
- (v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
© OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30
- (vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: Not Yet Known
(B) FILING DATE: Herewith
© CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: John P. White
(B) REGISTRATION NUMBER: 28,678
© REFERENCE/DOCKET NUMBER: 0575/50852-A
- (ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (212) 278-0400
(B) TELEFAX: (212) 391-0525
© TELEX:

(2) INFORMATION FOR SEQ ID NO:1:

- (I) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 3649 base pairs
(B) TYPE: nucleic acid
© STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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CCACCCACCT CGGCCTCCTA AAGTGCTGGG ATTACAGACA TGAGCCACCG CGCCCAGCCC      120
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ACAGTGAATT	TCTGATTTCA	CTCAGGGTGA	TAAATCAGAC	TCTTGGGGAA	GCGGGTGGTG	240
GCTCTGGACA	GCAGCAGGAA	TGGGGATCCA	GTTAGCAACA	AATCCATGGA	CCTATGACAG	300
GCTGAAAGCC	ACCCCTTCTC	CATCTTTGGG	AGGTTGCCAA	TGTCTGATTT	AACACTATCC	360
AATGAATGAT	CATTGAAAGT	AAAAAATAAC	TATCAACTAG	CAGAAAATAT	AAATGGTAAG	420
CATTAGCACA	TATTTACAT	GTTTATATTT	GGCTCTCAGA	TTGACCTATA	AAACAAAGTC	480
TGGGAAATTC	TATATGATCC	TGAAAAAATG	ATACGCTGGT	CTGGATGGTA	GAATAAGTTG	540
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TTCAGGATTT	TTGGTATACA	GGTGGTTTTT	GGTTACATGG	AAAAGTTCTT	TACTGGTGAT	660
TTCTGAGATT	TTAGTTCACC	CCTTATCCTG	AGCAGTGTAC	ACTGTTCCCA	ATATGTAGCC	720
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CCATTAAGAG	TGAATGTGTA	CCCTCCCTCT	AGCCTTTATT	ATTACTGTTT	TTGCTATTAC	900
ATGTGTTAGT	GTATGTGAAT	TTAATGCTTA	AAAATGTATC	CCATTGGCTA	CTATGGCAAA	960
AGGTTGACTC	ATAAGAGTTT	AGCACGGGTT	AAGATCTGAA	AGTTTTCTCC	CAGCCTCTTA	1020
TCACTGGCGC	AGACTTCACA	ATTCATGGAA	GCCACCACTG	AGATGACATT	GCCTCAGGCA	1080
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GGCCTCAGAC	AATACAATGG	TGGGTGAAGA	GAAGATGTCT	CTAAGAAACC	GGCTGTCAAA	1440
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GGACCACATC	AGAACAATAT	ATCACATGTT	TATTGCCCTC	CTCATCTCTC	TTATCCTCAG	1860
CACACTTGTA	GTAGATTACA	TTGATGAAGG	AAGGCTGGTG	CTTGAGTTCA	GCCTCCTGTC	1920
TTATGCTTTT	GGCAAATTTT	CTACCGTTGT	TTGGACCTGG	TGGATCATGT	TCCTGTCTAC	1980
ATTTTCAGTT	CCCTATTTTC	TGTTTCAACA	TTGGCGCACT	GGCTATAGCA	AGAGTTCTCA	2040

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AGGTTTTTGA CCAACATATG TTGTGTTAGC ATATACTG CCACCAGCTT CCCGGTTCAT	2160
CATTATATTC GAGCAGATTC GTTTTGTAAT GAAGGCCAC TCATTTGTCA GAGAGAACGT	2220
GCCTCGGGTA CTAAATTCAG CTAAGGAGAA ATCAAGCACT GTTCCAATAC CTACAGTCAA	2280
CCAGTATTTG TACTTCTTAT TTGCTCCTAC CTTATCTAC CGTGACAGCT ATCCCAGGAA	2340
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GCCCTTCAGC GCTCGTGTTT TGGTCCTATG TGGTATTTAA CTCCATCTTG CCAGGTGTGC	2520
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TGTTACGCTT TGGTGACAGG ATGTTCTATA AGGATTGGTG GAACTCCACG TCATACTCCA	2640
ACTATTATAG AACCTGGAAT GTGGTGGTCC ATGACTGGCT ATATTACTAT GCTTACAAGG	2700
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AGTTGTTACT GAAGTTATCT GTGTTATTTG GACCACTCCA GGCTTTACAG ATGACTCACT	3180
CCATTCCTAG GTCACCTGAA GCCAACTGT TGGAAGTTCA CTGGAGTCTT GTACACTTAA	3240
GCAGAGCAGA ACTTTTTTTT TGGGGCTGGG TGGGGGGAGA AGACCGACTA ACAGCTGAAG	3300
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CTATTTCTAG TAGAAAGAAA ATGTCTGTTT TCCAAAGATA ATGTTATACA TCCTATTTTG	3600
TAATTTTTTT GAAAAAGTT CAATGTTTCT TTTTCCTTAGT TTTTACCTT	3660

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 550 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

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(ii) MOLECULE TYPE: Amino Acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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Arg Glu Asn Pro Glu Glu Asp Glu Asp Gln Arg Asn Pro Ala Lys Glu
          20           25           30
Ser Leu Glu Thr Pro Ser Asn Gly Arg Ile Asp Ile Lys Gln Leu Ile
          35           40           45
Ala Lys Lys Ile Lys Leu Thr Ala Glu Ala Glu Glu Leu Lys Pro Phe
          50           55           60
Phe Met Lys Glu Val Gly Ser His Phe Asp Asp Phe Val Thr Asn Leu
          65           70           75           80
Ile Glu Lys Ser Ala Ser Leu Asp Asn Gly Gly Cys Ala Leu Thr Thr
          85           90           95
Phe Ser Val Leu Glu Gly Glu Lys Asn Asn His Arg Ala Lys Asp Leu
          100          105          110
Arg Ala Pro Pro Glu Gln Gly Lys Ile Phe Ile Ala Arg Arg Ser Leu
          115          120          125
Leu Asp Glu Leu Leu Glu Val Asp His Ile Arg Thr Ile Tyr His Met
          130          135          140
Phe Ile Ala Leu Leu Ile Leu Phe Ile Leu Ser Thr Leu Val Val Asp
          145          150          155          160
Tyr Ile Asp Glu Gly Arg Leu Val Leu Glu Phe Ser Leu Leu Ser Tyr
          165          170          175
Ala Phe Gly Lys Phe Pro Thr Val Val Trp Thr Trp Trp Ile Met Phe
          180          185          190
Leu Ser Thr Phe Ser Val Pro Tyr Phe Leu Phe Gln His Trp Arg Thr
          195          200          205
Gly Tyr Ser Lys Ser Ser His Pro Leu Ile Arg Ser Leu Phe His Gly
          210          215          220
Phe Leu Phe Met Ile Phe Gln Ile Gly Val Leu Gly Phe Gly Pro Thr
          225          230          235          240
Tyr Val Val Leu Ala Tyr Thr Leu Pro Pro Ala Ser Arg Phe Ile Ile
          245          250          255
Ile Phe Glu Gln Ile Arg Phe Val Met Lys Ala His Ser Phe Val Arg
          260          265          270
Glu Asn Val Pro Arg Val Leu Asn Ser Ala Lys Glu Lys Ser Ser Thr
          275          280          285
Val Pro Ile Pro Thr Val Asn Gln Tyr Leu Tyr Phe Leu Phe Ala Pro
          290          295          300

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Thr Leu Ile Tyr Arg Asp Ser Tyr Pro Arg Asn Pro Thr Val Arg Trp
 305 310 315 320
 Gly Tyr Val Ala Met Lys Phe Ala Gln Val Phe Gly Cys Phe Phe Tyr
 325 330 335
 Val Tyr Tyr Ile Phe Glu Arg Leu Cys Ala Pro Leu Phe Arg Asn Ile
 340 345 350
 Lys Gln Glu Pro Phe Ser Ala Arg Val Leu Val Leu Cys Val Phe Asn
 355 360 365
 Ser Ile Leu Pro Gly Val Leu Ile Leu Phe Leu Thr Phe Phe Ala Phe
 370 375 380
 Leu His Cys Trp Leu Asn Ala Phe Ala Glu Met Leu Arg Phe Gly Asp
 385 390 395 400
 Arg Met Phe Tyr Lys Asp Trp Trp Asn Ser Thr Ser Tyr Ser Asn Tyr
 405 410 415
 Tyr Arg Thr Trp Asn Val Val Val His Asp Trp Leu Tyr Tyr Tyr Ala
 420 425 430
 Tyr Lys Asp Phe Leu Trp Phe Phe Ser Lys Arg Phe Lys Ser Ala Ala
 435 440 445
 Met Leu Ala Val Phe Ala Val Ser Ala Val Val His Glu Tyr Ala Leu
 450 455 460
 Ala Val Cys Leu Ser Phe Phe Tyr Pro Val Leu Phe Val Leu Phe Met
 465 470 475 480
 Phe Phe Gly Met Ala Phe Asn Phe Ile Val Asn Asp Ser Arg Lys Lys
 485 490 495
 Pro Ile Trp Asn Val Leu Met Trp Thr Ser Leu Phe Leu Gly Asn Gly
 500 505 510
 Val Leu Leu Cys Phe Tyr Ser Gln Glu Trp Tyr Ala Arg Arg His Cys
 515 520 525
 Pro Leu Lys Asn Pro Thr Phe Leu Asp Tyr Val Arg Pro Arg Ser Trp
 530 535 540
 Thr Cys Arg Tyr Val Phe
 545 550

(2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 2601 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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GCCCTCCAGC TCTCTACTAA GACCGGTCGC AAGCATGCTG GGCGATATAT CCAAACCACA	60
CCACACATGG TCTCCCTCCT GCGTCAAAAT CTCCCAGAC AGTCCGGACC CGCACCCGAT	120
ATCCAGAATG AAAGTGCACG GCTGCAGATT CAAAAGCTCC AACGCCCTCA GCGTCATCTT	180
CGCCTGGATA TGCTGCACTC TGGTCGAACC CGTGTACTTG TGTGCTTCGC TATCATTATA	240
GAAAATCTCC GGTGGTGCCA ACTCCTCAGG ACGTGACATT ATTTCTTCTC TGATATATTT	300
CCTGTGTTTC CGTACCGCAC CTTTTTAGCA CTACTTTTTT ACTATGCTCT TCTTCTTCTG	360
CTTCTTCTGC TTTTTTCCTC TTTATCACAC TATGTATGTG CTGCTCATCT CTTCTTTTTA	420
TCGATAAAAT TGAAAAATGT GAGATGGTGT AGAGTGAAAA AAAAAAAAAA ATCTGGCTTG	480
GCCATCAAAT ACCCGGCCGT GGTTGGACTC GTTTAGCGAA CAATAGCACC CAGCAGACCC	540
TGGCAACATG CGGATGATAT AAGAAGGACG AGCGTGGTGG AGGAAAGGGG CGCCATTGGC	600
ACACTCACGC AGGTGGTTGT TCAGCACGGC TTGCAGCAAG AGCGCCAAAA CAGATTGCAA	660
GAATGACGGA GACTAAGGAT TTGTTGCAAG ACGAAGAGTT TCTTAAGATC CGCAGACTCA	720
ATTCCGCAGA AGCCAACAAA CGGCATTCCG TCACGTACGA TAACGTGATC CTGCCACAGG	780
AGTCCATGGA GGTTTCGCCA CGGTCGTCTA CCACGTCGCT GGTGGAGCCA GTGGAGTCGA	840
CTGAAGGAGT GGAGTCGACT GAGGCGGAAC GTGTGGCAGG GAAGCAGGAG CAGGAGGAGG	900
AGTACCCTGT GGACGCCAC ATGCAAAAGT ACCTTTCACA CCTGAAGAGC AAGTCTCGGT	960
CGAGGTTCCA CCGAAAGGAT GCTAGCAAGT ATGTGTCGTT TTTTGGGGAC GTGAGTTTGT	1020
ATCCTCGCCC CACGCTCCTG GACAGCGCCA TCAACGTGCC CTTCCAGACG ACTTTCAAAG	1080
GTCCGGTGCT GGAGAAACAG CTCAAAAATT TACAGTTGAC AAAGACCAAG ACCAAGGCCA	1140
CGGTGAAGAC TACGGTGAAG ACTACGGAGA AAACGGACAA GGCAGATGCC CCCCAGGAG	1200
AAAACTGGA GTCGAACTTT TCAGGGATCT ACGTGTCGC ATGGATGTTT TTGGGCTGGA	1260
TAGCCATCAG GTGCTGCACA GATTACTATG CGTCGTACGG CAGTGATGG AATAAGCTGG	1320
AAATCGTGCA GTACATGACA ACGGACTTGT TCACGATCGC AATGTTGGAC TTGGCAATGT	1380
TCCTGTGCAC TTTCTTCGTG GTTTTCGTGC ACTGGCTGGT GAAAAAGCGG ATCATCAACT	1440
GGAAGTGGAC TGGGTTTCGTT GCAGTGAGCA TCTTCGAGTT GGCTTTCATC CCCGTGACGT	1500
TCCCCATTTA CGTCTACTAC TTTGATTTCA ACTGGGTCAC GAGAATCTTC CTGTTCTGTC	1560
ACTCCGTGGT GTTTGTTATG AAGAGCCACT CGTTTGCCTT TTACAACGGG TATCTTTGGG	1620
ACATAAAGCA GGAAGTCGAG TACTCTTCCA AACAGTTGCA AAAATACAAG GAATCTTTGT	1680
CCCCAGAGAC CCGCGAGATT CTGCAAAAAA GTTGCGACTT TTGCCTTTTC GAATTGAACT	1740
ACCAGACCAA GGATAACGAC TTCCCCAACA ACATCAGTTG CAGCAATTTT TTCATGTTCT	1800
GTTTGTTCCC CGTCCTCGTG TACCAGATCA ACTACCCAAG AACGTCGCGC ATCAGATGGA	1860
GGTATGTGTT GGAGAAGGTG TCGCCATCA TTGGCACCAT CTTCTCATG ATGGTCACGG	1920

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CACAGTTCTT CATGCACCCG GTGGCCATGC GCTGTATCCA GTTCCACAAC ACGCCACCT 1980
TCGGCGGCTG GATCCCCGCC ACGCAAGAGT GGTTCCACCT GCTCTTCGAC ATGATTCCGG 2040
GCTTCACTGT TCTGTACATG CTCACGTTTT ACATGATATG GGACGCTTTA TTGAATTGCG 2100
TGGCGGAGTT GACCAGGTTT GCGGACAGAT ATTTCTACGG CGACTGGTGG AATTGCGTTT 2160
CGTTTGAAGA GTTTAGCAGA ATCTGGAACG TCCCCGTTCA CAAATTTTTA CTAAGACACG 2220
TGTACCACAG CTCCATGGGC GCATTGCATT TGAGCAAGAG CCAAGCTACA TTATTTACTT 2280
TTTTCTTGAG TGCCGTGTTC CACGAAATGG CCATGTTTCGC CATTTTCAGA AGGGTTAGAG 2340
GATATCTGTT CATGTTCCAA CTGTCGCAGT TTGTGTGGAC TGCTTTGAGC AACACCAAGT 2400
TTCTACCGGC AAGACCGCAG TTGTCCAACG TTGTCTTTTC GTTTGGTGTC GTTTCAGGGC 2460
CCAGTATCAT TATGACGTTG TACCTGACCT TATGAACTGC CACCATACCA CGTGTGTCCC 2520
TCGCAAGCCC TTGATAGATA TACAATAGGG AATGGGCGTC CGTCCACCGT GGTCAAAGAC 2580
AGGGGCAAAG AGCTCCTAGG T

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 610 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Amino Acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Met Thr Glu Thr Lys Asp Leu Leu Gln Asp Glu Glu Phe Leu Lys Ile
1 5 10 15
Arg Arg Leu Asn Ser Ala Glu Ala Asn Lys Arg His Ser Val Thr Tyr
20 25 30
Asp Asn Val Ile Leu Pro Gln Glu Ser Met Glu Val Ser Pro Arg Ser
35 40 45
Ser Thr Thr Ser Leu Val Glu Pro Val Glu Ser Thr Glu Gly Val Glu
50 55 60
Ser Thr Glu Ala Glu Arg Val Ala Gly Lys Gln Glu Gln Glu Glu Glu
65 70 75 80
Tyr Pro Val Asp Ala His Met Gln Lys Tyr Leu Ser His Leu Lys Ser
85 90 95
Lys Ser Arg Ser Arg Phe His Arg Lys Asp Ala Ser Lys Tyr Val Ser
100 105 110
Phe Phe Gly Asp Val Ser Phe Asp Pro Arg Pro Thr Leu Leu Asp Ser
115 120 125

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Ala Ile Asn Val Pro Phe Gln Thr Thr Phe Lys Gly Pro Val Leu Glu
 130 135 140
 Lys Gln Leu Lys Asn Leu Gln Leu Thr Lys Thr Lys Thr Lys Ala Thr
 145 150 155 160
 Val Lys Thr Thr Val Lys Thr Thr Glu Lys Thr Asp Lys Ala Asp Ala
 165 170 175
 Pro Pro Gly Glu Lys Leu Glu Ser Asn Phe Ser Gly Ile Tyr Val Phe
 180 185 190
 Ala Trp Met Phe Leu Gly Trp Ile Ala Ile Arg Cys Cys Thr Asp Tyr
 195 200 205
 Tyr Ala Ser Tyr Gly Ser Ala Trp Asn Lys Leu Glu Ile Val Gln Tyr
 210 215 220
 Met Thr Thr Asp Leu Phe Thr Ile Ala Met Leu Asp Leu Ala Met Phe
 225 230 235 240
 Leu Cys Thr Phe Phe Val Val Phe Val His Trp Leu Val Lys Lys Arg
 245 250 255
 Ile Ile Asn Trp Lys Trp Thr Gly Phe Val Ala Val Ser Ile Phe Glu
 260 265 270
 Leu Ala Phe Ile Pro Val Thr Phe Pro Ile Tyr Val Tyr Tyr Phe Asp
 275 280 285
 Phe Asn Trp Val Thr Arg Ile Phe Leu Phe Leu His Ser Val Val Phe
 290 295 300
 Val Met Lys Ser His Ser Phe Ala Phe Tyr Asn Gly Tyr Leu Trp Asp
 305 310 315 320
 Ile Lys Gln Glu Leu Glu Tyr Ser Ser Lys Gln Leu Gln Lys Tyr Lys
 325 330 335
 Glu Ser Leu Ser Pro Glu Thr Arg Glu Ile Leu Gln Lys Ser Cys Asp
 340 345 350
 Phe Cys Leu Phe Glu Leu Asn Tyr Gln Thr Lys Asp Asn Asp Phe Pro
 355 360 365
 Asn Asn Ile Ser Cys Ser Asn Phe Phe Met Phe Cys Leu Phe Pro Val
 370 375 380
 Leu Val Tyr Gln Ile Asn Tyr Pro Arg Thr Ser Arg Ile Arg Trp Arg
 385 390 395 400
 Tyr Val Leu Glu Lys Val Cys Ala Ile Ile Gly Thr Ile Phe Leu Met
 405 410 415
 Met Val Thr Ala Gln Phe Phe Met His Pro Val Ala Met Arg Cys Ile
 420 425 430
 Gln Phe His Asn Thr Pro Thr Phe Gly Gly Trp Ile Pro Ala Thr Gln
 435 440 445
 Glu Trp Phe His Leu Leu Phe Asp Met Ile Pro Gly Phe Thr Val Leu
 450 455 460

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Tyr Met Leu Thr Phe Tyr Met Ile Trp Asp Ala Leu Leu Asn Cys Val
 465 470 475 480
 Ala Glu Leu Thr Arg Phe Ala Asp Arg Tyr Phe Tyr Gly Asp Trp Trp
 485 490 495
 Asn Cys Val Ser Phe Glu Glu Phe Ser Arg Ile Trp Asn Val Pro Val
 500 505 510
 His Lys Phe Leu Leu Arg His Val Tyr His Ser Ser Met Gly Ala Leu
 515 520 525
 His Leu Ser Lys Ser Gln Ala Thr Leu Phe Thr Phe Phe Leu Ser Ala
 530 535 540
 Val Phe His Glu Met Ala Met Phe Ala Ile Phe Arg Arg Val Arg Gly
 545 550 555 560
 Tyr Leu Phe Met Phe Gln Leu Ser Gln Phe Val Trp Thr Ala Leu Ser
 565 570 575
 Asn Thr Lys Phe Leu Arg Ala Arg Pro Gln Leu Ser Asn Val Val Phe
 580 585 590
 Ser Phe Gly Val Cys Ser Gly Pro Ser Ile Ile Met Thr Leu Tyr Leu
 595 600 605
 Thr Leu
 610

(2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 2421 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

TATAAAATTC CTTTCATCAA TACATCTATA TATTCGAATA TATAGATAAA CCAATACAAA	60
AACATACTGA AATTTTTTTGA AAACAACATA AACTATTCAT TGCAGTTACA CGTGAATGCT	120
AAACTTTATA TCGCTCTTGT CGGTCCCGCG GAGTTAACAT TTAACGGCTT CTCGCGCAAT	180
AACCGGAAAA ATTCCAACAG TTTCTTTGTA ATATTATTAA GCCTTCTTTT TTCCCGGAAT	240
CTATAAGAGG GGACGAAAAT TAGCCGCTAT TAATTCTGGT ATTGCCACCT AGACAAGAAG	300
TAAACAGACA CATTACGTTA GCAAAAGCAA CAATAACAAA CACAACCATG GACAAGAAGA	360
AGGATCTACT GGAGAACGAA CAATTTCTCC GCATCCAAAA GCTCAACGCT GCCGATGCGG	420
GCAAAAGACA ATCTATAACA GTGGACGACG AGGGCGAACT ATATGGGTTA GACACCTCCG	480
GCAACTCACC AGCCAATGAA CACACAGCTA CCACAATTAC ACAGAATCAC AGCGTGGTGG	540

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CCTCAAACGG AGACGTCGCA TTCATCCCAG GAACTGCTAC CGAAGGCAAT ACAGAGATTG	600
TAACTGAAGA AGTGATTGAG ACCGATGATA ACATGTTCAA GACCCATGTG AAGACTTTAA	660
GCTCCAAAGA GAAGGCACGG TATAGGCAAG GGTCTCTAA CTTTATATCG TATTTGATG	720
ATATGTCATT TGAACACAGG CCCAGTATAT TAGATGGGTC AGTTAACGAG CCCTTCAAGA	780
CCAAATTCGT GGGACCTACT TTAGAAAAGG AGATCAGAAG AGGGGAGAAA GAGCTAATGG	840
CCATGCGCAA AAATTTACAC CACCGCAAGT CCTCCCCAGA TGCTGTCGAC TCAGTAGGGA	900
AAAATGATGG CGCCGCCCA ACTACTGTTC CAACTGCCGC CACCTCAGAA ACGGTGGTCA	960
CCGTTGAAAC CACCATAATT TCATCCAATT TCTCCGGGTT GTACGTGGCG TTTTGGATGG	1020
CTATTGCATT TGGTGCTGTC AAGGCTTTAA TAGACTATTA TTACCAGCAT AATGGTAGCT	1080
TCAAGGATTC GGAGATCTTG AAATTTATGA CTACGAATTT GTTCACTGTG GCATCCGTAG	1140
ATCTTTTGAT GTATTTGAGC ACTTATTTTG TCGTTGGAAT ACAATACTTA TGCAAGTGGG	1200
GGGTCTTGAA ATGGGGCACT ACCGGCTGGA TCTTCACCTC AATTTACGAG TTTTGTGTTG	1260
TTATCTTCTA CATGTATTTA ACAGAAAACA TCCTAAACT ACACTGGCTG TCCAAGATCT	1320
TCCTTTTTTT GCATTCTTTA GTTTTATTGA TGAAAATGCA TTCTTTCGCC TTCTACAATG	1380
GCTATCTATG GGGTATAAAG GAAGAACTAC AATTTTCCAA AAGCGCTCTT GCCAAATACA	1440
AGGATTCTAT AAATGATCCA AAAGTTATTG GTGCTCTTGA GAAAAGCTGT GAGTTTTGTA	1500
GTTTTGAATT GAGCTCTCAG TCTTTAAGCG ACCAACTCA AAAATTCCCC AACAATATCA	1560
GTGCAAAAAG CTTTTTTTGG TTCACCATGT TTCCAACCTT AATTTACCAA ATTGAATATC	1620
CAAGAACTAA GGAAATCAGA TGGAGCTACG TATTAGAAAA GATCTGCGCC ATCTTCGGTA	1680
CCATTTTCTT AATGATGATA GATGCTCAA TCTTGATGTA TCCTGTAGCA ATGAGAGCAT	1740
TGGCTGTGCG CAATTCTGAA TGGACTGGTA TATTGGATAG ATTATTGAAA TGGGTTGGAT	1800
TGCTCGTTGA TATCGTCCCA GGGTTTATCG TGATGTACAT CTTGGACTTC TATTTGATTT	1860
GGGATGCCAT TTTGAACTGT GTGGCTGAAT TGACAAGATT TGGCGACAGA TATTTCTACG	1920
GTGACTGGTG GAATTGTGTT AGTTGGGCAG ACTTCAGTAG AATTTGGAAC ATCCCAGTGC	1980
ATAAGTTTTT GTTAAGACAT GTTTACCATA GTTCAATGAG TTCATTCAAA TTGAACAAGA	2040
GTCAAGCAAC TTTGATGACC TTTTCTTAA GTTCCGTCGT TCATGAATTA GCAATGTACG	2100
TTATCTTCAA GAAATTGAGG TTTTACTTGT TCTTCTTCCA AATGCTGCAA ATGCCATTAG	2160
TAGCTTTAAC AAATACTAAA TTCATGAGGA ACAGAACCAT AATCGGAAAT GTTATTTTCT	2220
GGCTCGGTAT CTGCATGGGA CCAAGTGTCA TGTGTACGTT GTACTTGACA TTCTAAGGCA	2280
TCCTGCAACT GTTCTGTGGA GCTATTAAAT CTTTATAGTA AATTTTTTTT TACTTTTTTT	2340
TTTTTTTTTT TTTTTTTT TATTTTACAA GCGTCTATAT ATTTTCTATT ATAGAATATT	2400
GTCATTTATT ACATTGGTTC A	

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(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 642 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Amino Acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

```

Met Asp Lys Lys Lys Asp Leu Leu Glu Asn Glu Gln Phe Leu Arg Ile
1      5      10      15
Gln Lys Leu Asn Ala Ala Asp Ala Gly Lys Arg Gln Ser Ile Thr Val
20      25      30
Asp Asp Glu Gly Glu Leu Tyr Gly Leu Asp Thr Ser Gly Asn Ser Pro
35      40      45
Ala Asn Glu His Thr Ala Thr Thr Ile Thr Gln Asn His Ser Val Val
50      55      60
Ala Ser Asn Gly Asp Val Ala Phe Ile Pro Gly Thr Ala Thr Glu Gly
65      70      75      80
Asn Thr Glu Ile Val Thr Glu Glu Val Ile Glu Thr Asp Asp Asn Met
85      90      95
Phe Lys Thr His Val Lys Thr Leu Ser Ser Lys Glu Lys Ala Arg Tyr
100     105     110
Arg Gln Gly Ser Ser Asn Phe Ile Ser Tyr Phe Asp Asp Met Ser Phe
115     120     125
Glu His Arg Pro Ser Ile Leu Asp Gly Ser Val Asn Glu Pro Phe Lys
130     135     140
Thr Lys Phe Val Gly Pro Thr Leu Glu Lys Glu Ile Arg Arg Arg Glu
145     150     155     160
Lys Glu Leu Met Ala Met Arg Lys Asn Leu His His Arg Lys Ser Ser
165     170     175
Pro Asp Ala Val Asp Ser Val Gly Lys Asn Asp Gly Ala Ala Pro Thr
180     185     190
Thr Val Pro Thr Ala Ala Thr Ser Glu Thr Val Val Thr Val Glu Thr
195     200     205
Thr Ile Ile Ser Ser Asn Phe Ser Gly Leu Tyr Val Ala Phe Trp Met
210     215     220
Ala Ile Ala Phe Gly Ala Val Lys Ala Leu Ile Asp Tyr Tyr Tyr Gln
225     230     235     240
His Asn Gly Ser Phe Lys Asp Ser Glu Ile Leu Lys Phe Met Thr Thr
245     250     255

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Asn Leu Phe Thr Val Ala Ser Val Asp Leu Leu Met Tyr Leu Ser Thr
 260 265 270
 Tyr Phe Val Val Gly Ile Gln Tyr Leu Cys Lys Trp Gly Val Leu Lys
 275 280 285
 Trp Gly Thr Thr Gly Trp Ile Phe Thr Ser Ile Tyr Glu Phe Leu Phe
 290 295 300
 Val Ile Phe Tyr Met Tyr Leu Thr Glu Asn Ile Leu Lys Leu His Trp
 305 310 315 320
 Leu Ser Lys Ile Phe Leu Phe Leu His Ser Leu Val Leu Leu Met Lys
 325 330 335
 Met His Ser Phe Ala Phe Tyr Asn Gly Tyr Leu Trp Gly Ile Lys Glu
 340 345 350
 Glu Leu Gln Phe Ser Lys Ser Ala Leu Ala Lys Tyr Lys Asp Ser Ile
 355 360 365
 Asn Asp Pro Lys Val Ile Gly Ala Leu Glu Lys Ser Cys Glu Phe Cys
 370 375 380
 Ser Phe Glu Leu Ser Ser Gln Ser Leu Ser Asp Gln Thr Gln Lys Phe
 385 390 395 400
 Pro Asn Asn Ile Ser Ala Lys Ser Phe Phe Trp Phe Thr Met Phe Pro
 405 410 415
 Thr Leu Ile Tyr Gln Ile Glu Tyr Pro Arg Thr Lys Glu Ile Arg Trp
 420 425 430
 Ser Tyr Val Leu Glu Lys Ile Cys Ala Ile Phe Gly Thr Ile Phe Leu
 435 440 445
 Met Met Ile Asp Ala Gln Ile Leu Met Tyr Pro Val Ala Met Arg Ala
 450 455 460
 Leu Ala Val Arg Asn Ser Glu Trp Thr Gly Ile Leu Asp Arg Leu Leu
 465 470 475 480
 Lys Trp Val Gly Leu Leu Val Asp Ile Val Pro Gly Phe Ile Val Met
 485 490 495
 Tyr Ile Leu Asp Phe Tyr Leu Ile Trp Asp Ala Ile Leu Asn Cys Val
 500 505 510
 Ala Glu Leu Thr Arg Phe Gly Asp Arg Tyr Phe Tyr Gly Asp Trp Trp
 515 520 525
 Asn Cys Val Ser Trp Ala Asp Phe Ser Arg Ile Trp Asn Ile Pro Val
 530 535 540
 His Lys Phe Leu Leu Arg His Val Tyr His Ser Ser Met Ser Ser Phe
 545 550 555 560
 Lys Leu Asn Lys Ser Gln Ala Thr Leu Met Thr Phe Phe Leu Ser Ser
 565 570 575
 Val Val His Glu Leu Ala Met Tyr Val Ile Phe Lys Lys Leu Arg Phe
 580 585 590

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Tyr Leu Phe Phe Phe Gln Met Leu Gln Met Pro Leu Val Ala Leu Thr
 595 600 605

Asn Thr Lys Phe Met Arg Asn Arg Thr Ile Ile Gly Asn Val Ile Phe
 610 615 620

Trp Leu Gly Ile Cys Met Gly Pro Ser Val Met Cys Thr Leu Tyr Leu
 625 630 635 640

Thr Phe

(2) INFORMATION FOR SEQ ID NO:7:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 983 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

ATGGAGCTCA ACTTTCCCCG CTCTCCCCGC ATCCGGAAGC GCTTTCTGCT GCGACGGATC 60

CTTGAGATGC TGTTCCTCAC CCAGCTCCAG GTGGGGCTGA TCCAGCAGTG GATGGTCCCC 120

ACCATCCAGA ACTCCATGAA GCCCTTCAAG GACATGGACT ACTCACGCAT CATCGAGCGC 180

CTCCTGAAGC TGGCGGTCCC CAATCACCTC ATCTGGCTCA TCTTCTTCTA CTGGCTCTTC 240

CACTCCTGCC TGAATGCCGT GGCTGAGCTC ATGCAGTTTG GAGACCGGGA GTTCTACCGG 300

GACTGGTGGA ACTCCGAGTC TGTCACCTAC TTCTGGCAGA ACTGGAACAT CCCTGTGCAC 360

AAGTGGTGCA TCAGACACTT CTACAAGCCC ATGCTTCGAC GGGGCAGCAG CAAGTGGATG 420

GCCAGGACAG GGGTGTTCCT GGCCTCGGCT TTCTTCCACG AGTACCTGGT GAGCGTCCCT 480

CTGCGAATGT TCCGCCTCTG GGCTTTCACG GGCATGATGG CTCAGATCCC ACTGGCCTGG 540

TTCGTGGGCC GCTTTTTCCTA GGGCAACTAT GGCAACGCAG CTGTGTGGCT GTCGCTCATC 600

ATCGGACAGC CAATAGCCGT CCTCATGTAC GTCCACGAAC TACTACGTGC TCAACTATGA 660

GGCCCCAGCG GCAGAGGCCT GAGCTGCACC TGAGGGCCTG GCTTCTCACT GCCACCTCAA 720

ACCCGCTGCC AGAGCCCACC TCTCCTCCTA GGCCTCGAGT GCTGGGGATG GGCCTGGCTG 780

CACAGCATCC TCCTCTGGTC CCAGGGAGGC CTCTCTGCCC TATGGGGCTC TGTCTTGCAC 840

CCCTCAGGGA TGGCGACAGC AGGCCAGACA CAGTCTGATG CCAGCTGGGA GTCTTGCTGA 900

CCCTGCCCCG GGTCCGAGGG TGTCATAAAA GTGCTGTCCA GTGAGAAAAA GAAAAAAAAA 960

AAAAAAAAA ATTCTGCGGC CGC

(2) INFORMATION FOR SEQ ID NO:8:

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(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 219 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Amino Acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

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Met Glu Leu Asn Phe Pro Arg Ser Pro Arg Ile Arg Lys Arg Phe Leu
 1           5           10           15
Leu Arg Arg Ile Leu Glu Met Leu Phe Phe Thr Gln Leu Gln Val Gly
          20           25           30
Leu Ile Gln Gln Trp Met Val Pro Thr Ile Gln Asn Ser Met Lys Pro
          35           40           45
Phe Lys Asp Met Asp Tyr Ser Arg Ile Ile Glu Arg Leu Leu Lys Leu
          50           55           60
Ala Val Pro Asn His Leu Ile Trp Leu Ile Phe Phe Tyr Trp Leu Phe
          65           70           75           80
His Ser Cys Leu Asn Ala Val Ala Glu Leu Met Gln Phe Gly Asp Arg
          85           90           95
Glu Phe Tyr Arg Asp Trp Trp Asn Ser Glu Ser Val Thr Tyr Phe Trp
          100          105          110
Gln Asn Trp Asn Ile Pro Val His Lys Trp Cys Ile Arg His Phe Tyr
          115          120          125
Lys Pro Met Leu Arg Arg Gly Ser Ser Lys Trp Met Ala Arg Thr Gly
          130          135          140
Val Phe Leu Ala Ser Ala Phe Phe His Glu Tyr Leu Val Ser Val Pro
          145          150          155          160
Leu Arg Met Phe Arg Leu Trp Ala Phe Thr Gly Met Met Ala Gln Ile
          165          170          175
Pro Leu Ala Trp Phe Val Gly Arg Phe Phe Gln Gly Asn Tyr Gly Asn
          180          185          190
Ala Ala Val Trp Leu Ser Leu Ile Ile Gly Gln Pro Ile Ala Val Leu
          195          200          205
Met Tyr Val His Glu Leu Leu Arg Ala Gln Leu
          210          215

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(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 455 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

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(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

ATGTTGAACT TCATGATGCA TGACCAGCGC ACCGGCCCCG CATGGAACGT GCTGATGTGG	60
ACCATGCTGT TTCTAGGCCA GGAATCCAG GTCAGCCTGT ACTGCCAGGA GTGGTACGCA	120
CGGACGCACT GCCCCCTTACC CCAGGCAACT TTCTGGGGGC TGGTGACACC TCGATCTTGG	180
TCCTGCCATA CCTAGAGGTC GGGACAGACG ACGCTACCTG CCCAGACACC ACCAAGTTCT	240
CTGCCTGCAA AACCTGGGGA CCAGGACTTC CTGTCTTGCA TTCCCAAATT TGGGTTCTTG	300
AGTCGAGGCA ACCTTGACACA CAAGACCCCA CCAAGGGATT GTTGCAAGGG ATTAGATTTT	360
GCAGATTTGT TGGGTAATGA TTCAACGACT CAGCTGGGGG TTGACCAGGG TTGATTTTTC	420
AATCCTTTTC CCCTGGGTTT GGGTTACAGG TTTT	455

(2) INFORMATION FOR SEQ ID NO:10:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 64 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Amino Acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Met	Leu	Asn	Phe	Met	Met	His	Asp	Gln	Arg	Thr	Gly	Pro	Ala	Trp	Asn	1	5	10	15
Val	Leu	Met	Trp	Thr	Met	Leu	Phe	Leu	Gly	Gln	Gly	Ile	Gln	Val	Ser	20	25	30	
Leu	Tyr	Cys	Gln	Glu	Trp	Tyr	Ala	Arg	Thr	His	Cys	Pro	Leu	Pro	Gln	35	40	45	
Ala	Thr	Phe	Trp	Gly	Leu	Val	Thr	Pro	Arg	Ser	Trp	Ser	Cys	His	Thr	50	55	60	

(2) INFORMATION FOR SEQ ID NO:11:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 517 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

-71-

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

```

ATGGACAACG CGGGGTCTGA TACGACTCAC TATAGGGAAT TTGGCCCTCG AGCAGTAGAT      60
TCGGCACGAT GGGCACGAGG ACTCCATCAT GTTCCTCAAG CTTTATTCCT ACCGGGATGT      120
CAACCTGTGG TGCCGCCAGC GAAGGGTCAA GGCCAAAGCT GTCTCTACAG GGAAGAAGGT      180
CAGTGGGGCT GCTGCGAGCA AGCTGTGAGC TATCCAGACA ACCTGACCTA CCGAGATCTC      240
GATTACTTCA TCTTTGCTCC TACTTTGTGT TATGAACTCA ACTTTCCTCG GTCCCCCGA      300
ATACGAGAGC GCTTTCTGCT ACGACGAGTT CTTGAGATGC TCTTTTTTAC CCAGCTTCAA      360
GTGGGGCTGA TCCAACAGTG GATGGTCCCT ACTATCCAGA ACTCCATGGA AGCCCTTTCA      420
AGAGCTTCTG GCAGTTTGG AGACGCGAG TTCTACAGAG ATTGGTGGAA TGCTGAGTCT      480
GTCACCGACT TTTGGCAGAA CTGGAATATC CCCGTGG      517

```

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 172 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Amino Acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

```

Met Asp Asn Ala Gly Ser Asp Thr Thr His Tyr Arg Glu Phe Gly Pro
1          5          10          15
Arg Ala Val Asp Ser Ala Arg Trp Ala Arg Gly Leu His His Val Pro
20          25          30
Gln Ala Leu Phe Leu Pro Gly Cys Gln Pro Val Val Pro Pro Ala Lys
35          40          45
Gly Gln Gly Gln Ser Cys Leu Tyr Arg Glu Glu Gly Gln Trp Gly Cys
50          55          60
Cys Glu Gln Ala Val Ser Tyr Pro Asp Asn Leu Thr Tyr Arg Asp Leu
65          70          75          80
Asp Tyr Phe Ile Phe Ala Pro Thr Leu Cys Tyr Glu Leu Asn Phe Pro
85          90          95
Arg Ser Pro Arg Ile Arg Glu Arg Phe Leu Leu Arg Arg Val Leu Glu
100         105         110
Met Leu Phe Phe Thr Gln Leu Gln Val Gly Leu Ile Gln Gln Trp Met
115         120         125
Val Pro Thr Ile Gln Asn Ser Met Glu Ala Leu Ser Arg Ala Ser Gly
130         135         140
Ser Phe Gly Asp Arg Glu Phe Tyr Arg Asp Trp Trp Asn Ala Glu Ser

```

-72-

145		150		155		160
Val Thr Asp Phe Trp Gln Asn Trp Asn Ile Pro Val						
		165		170		

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 366 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Amino Acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

Met	Lys	Asp	Leu	Leu	Glu	Phe	Leu	Lys	Ile	Arg	Leu	Asn	Ala	Asp	Ala
1				5					10					15	
Lys	Arg	Ser	Thr	Asp	Ser	Pro	Thr	Val	Ser	Glu	Val	Glu	Arg	Gly	Lys
			20					25					30		
Gln	Glu	Ile	Glu	Ala	His	Lys	Ser	Lys	Lys	Arg	Phe	Arg	Ser	Phe	Ser
		35					40					45			
Phe	Phe	Asp	Ser	Phe	Glu	Arg	Pro	Ser	Leu	Leu	Asp	Gly	Asn	Pro	Phe
	50					55					60				
Thr	Thr	Phe	Gly	Pro	Val	Leu	Glu	Lys	Glu	Lys	Asn	Leu	His	Lys	Lys
65					70				75					80	
Lys	Thr	Thr	Val	Thr	Asp	Val	Ser	Asn	Phe	Ser	Gly	Ile	Tyr	Val	Phe
				85					90					95	
Trp	Met	Leu	Ala	Leu	Asp	Tyr	Tyr	Gly	Glu	Ile	Leu	Tyr	Met	Thr	Thr
			100					105					110		
Leu	Phe	Thr	Val	Ala	Asp	Leu	Met	Phe	Leu	Ser	Thr	Phe	Phe	Val	Val
		115					120					125			
Leu	Lys	Trp	Thr	Gly	Ile	Ser	Ile	Glu	Phe	Leu	Phe	Ile	Phe	Leu	Trp
	130					135					140				
Ser	Arg	Ile	Phe	Leu	Phe	Leu	His	Ser	Val	Phe	Val	Met	Lys	His	Ser
145					150					155				160	
Phe	Ala	Phe	Tyr	Asn	Gly	Tyr	Leu	Trp	Ile	Lys	Glu	Glu	Leu	Ser	Leu
				165					170					175	
Lys	Tyr	Lys	Glu	Ser	Ser	Pro	Leu	Gln	Lys	Ser	Cys	Phe	Cys	Phe	Glu
			180					185					190		
Leu	Gln	Phe	Pro	Asn	Asn	Ile	Ser	Phe	Phe	Phe	Phe	Pro	Thr	Leu	Ile
		195					200					205			
Tyr	Gln	Ile	Tyr	Pro	Arg	Thr	Ile	Arg	Trp	Tyr	Val	Leu	Glu	Lys	Cys
	210					215					220				
Ala	Ile	Phe	Gly	Thr	Ile	Phe	Leu	Met	Met	Ala	Gln	Met	Pro	Val	Ala

-73-

225		230		235		240
Met Arg Asn Phe Trp Gln Leu Leu Asp Ile Pro Gly Phe Val Leu Tyr						
		245		250		255
Leu Thr Phe Tyr Ile Trp Asp Ala Leu Asn Cys Val Ala Glu Leu Thr						
		260		265		270
Arg Phe Gly Asp Arg Tyr Phe Tyr Gly Asp Trp Trp Asn Cys Val Ser						
		275		280		285
Phe Ser Arg Ile Trp Asn Val Pro Val His Lys Phe Leu Leu Arg His						
		290		295		300
Val Tyr His Ser Ser Met Phe Lys Leu Lys Ser Gln Ala Thr Leu Thr						
		305		310		315
Phe Phe Leu Ser Ala Val Val His Glu Ala Met Val Ile Phe Arg Tyr						
		325		330		335
Leu Phe Phe Gln Gln Met Ala Leu Asn Thr Lys Phe Arg Arg Ile Asn						
		340		345		350
Val Phe Trp Gly Cys Gly Pro Ser Val Thr Leu Tyr Leu Thr						
		355		360		365

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 96 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Amino Acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

Pro Asn His Leu Ile Trp Leu Ile Phe Phe Tyr Trp Leu Phe His Ser														
1			5				10						15	
Cys Leu Asn Ala Val Ala Glu Leu Met Gln Phe Gly Asp Arg Glu Phe														
			20				25						30	
Tyr Arg Asp Trp Trp Asn Ser Glu Ser Val Thr Tyr Phe Trp Gln Asn														
			35				40						45	
Trp Lys Ile Pro Val His Lys Trp Cys Ile Arg His Phe Tyr Lys Pro														
			50				55						60	
Met Leu Arg Arg Gly Ser Ser Lys Trp Met Ala Arg Asp Arg Gly Val														
			65				70						75	
Pro Gly Pro Ser Ala Phe Phe His Val Val Thr Trp Val Ser Val Pro														
							85						90	
														95

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

-74-

- (A) LENGTH: 91 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

GAGGGGACGA AAATTAGCCG CTATTAATTC TGGTATTGCC ACCTAGACAA GAAGTAAACA 60
GACACAGATG CAAGAGTTCG AATCTCTTAG C 91

(2) INFORMATION FOR SEQ ID NO:16:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 76 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

CTATAAAGAT TTAATAGCTC CACAGAACAG TTGCAGGATG CCTTAGGGTC GACTACGTCG 60
TAAGGCCGTT TCTGAC 76

(2) INFORMATION FOR SEQ ID NO:17:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 22 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

CATTGCAGTT ACACGTGAAT GC 22

(2) INFORMATION FOR SEQ ID NO:18:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 23 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

-75-

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

TAGCTCCACA GAACAGTTGC AGG

23

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

CTCTGACAAC AACGAAGTCA G

21

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What is claimed is:

1. An isolated nucleic acid which encodes an acylcoenzyme A: cholesterol acyltransferase II.
5
2. An isolated nucleic acid which encodes an acylcoenzyme A: cholesterol acyltransferase III.
3. The isolated nucleic acid of claim 1 or 2, wherein
10 the nucleic acid is DNA or RNA.
4. The isolated nucleic acid of claim 3, wherein the nucleic acid is cDNA or genomic DNA.
- 15 5. The isolated nucleic acid of claim 1 comprising a nucleic acid having the sequence as set forth in Figure 15.
- 20 6. The isolated nucleic acid of claim 1, wherein the nucleic acid encodes a human wildtype acylcoenzyme A: cholesterol acyltransferase II having substantially the same amino acid sequence as set forth in Figure 15.
- 25 7. The isolated nucleic acid of claim 2, comprising a nucleic acid having the sequence as set forth in Figure 16.
- 30 8. The isolated nucleic acid of claim 2, wherein the nucleic acid encodes a human wildtype acylcoenzyme A: cholesterol acyltransferase III having substantially the same amino acid sequence as set forth in Figure 16.
- 35 9. The isolated nucleic acid of claim 1 comprising a

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nucleic acid having the sequence designated Seq. I.D. No.: 11.

- 5 10. The isolated nucleic acid of claim 1, wherein the nucleic acid encodes a mouse wildtype acylcoenzyme A: cholesterol acyltransferase II having substantially the same amino acid sequence as the sequence designated Seq. I.D. No.: 12.
- 10 11. The isolated nucleic acid of claim 1, wherein the nucleic acid encodes a mutant acylcoenzyme A: cholesterol acyltransferase II.
- 15 12. The isolated nucleic acid of claim 2, wherein the nucleic acid encodes a mutant acylcoenzyme A: cholesterol acyltransferase III.
- 20 13. A vector comprising the isolated nucleic acid of claim 1 or 2.
- 25 14. The vector of claim 13 further comprising a promoter of RNA transcription operatively linked to the nucleic acid.
- 30 15. The vector of claim 14, wherein the promoter comprises a bacterial, yeast, insect or mammalian promoter.
16. The vector of claim 14, further comprising plasmid, cosmid, yeast artificial chromosome (YAC), bacteriophage or eukaryotic viral DNA.
- 35 17. The vector of claim 14 designated YEpAB-ACAT2.
18. A host vector system for the production of a

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polypeptide which comprises the vector of claim 14 in a suitable host.

- 5 19. The host vector system of claim 18, wherein the suitable host is a prokaryotic or eukaryotic cell.
20. The host vector system of claim 19, wherein the prokaryotic cell is a bacterial cell.
- 10 21. The host vector system of claim 19, wherein the eukaryotic cell is a yeast, insect, plant or mammalian cell.
- 15 22. A method for producing a polypeptide which comprises growing the host vector system of claim 18 under suitable conditions permitting production of the polypeptide and recovering the polypeptide so produced.
- 20 23. A method of obtaining a polypeptide in purified form which comprises:
- (a) introducing the vector of claim 14 into a suitable host cell;
 - (b) culturing the resulting cell so as to produce the polypeptide;
 - 25 © recovering the polypeptide produced in step (b); and
 - (d) purifying the polypeptide so recovered.
- 30 24. A purified wildtype acylcoenzyme A: cholesterol acyltransferase II.
25. A purified mutant acylcoenzyme A: cholesterol acyltransferase II.

35

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26. A purified wildtype acylcoenzyme A: cholesterol acyltransferase III.
27. A purified mutant acylcoenzyme A: cholesterol acyltransferase III.
28. An oligonucleotide of at least 15 nucleotides capable of specifically hybridizing with a unique sequence of nucleotides present within a nucleic acid which encodes a wildtype acylcoenzyme A: cholesterol acyltransferase II without hybridizing to a nucleic acid which encodes a mutant acylcoenzyme A: cholesterol acyltransferase II.
29. An oligonucleotide of at least 15 nucleotides capable of specifically hybridizing with a unique sequence of nucleotides present within the nucleic acid which encodes a mutant acylcoenzyme A: cholesterol acyltransferase II without hybridizing to a nucleic acid which encodes a wildtype acylcoenzyme A: cholesterol acyltransferase II.
30. An oligonucleotide of at least 15 nucleotides capable of specifically hybridizing with a unique sequence of nucleotides present within a nucleic acid which encodes a wildtype acylcoenzyme A: cholesterol acyltransferase III without hybridizing to a nucleic acid which encodes a mutant acylcoenzyme A: cholesterol acyltransferase III.
31. An oligonucleotide of at least 15 nucleotides capable of specifically hybridizing with a unique sequence of nucleotides present within the nucleic acid which encodes a mutant acylcoenzyme A: cholesterol acyltransferase III without hybridizing

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to a nucleic acid which encodes a wildtype acylcoenzyme A: cholesterol acyltransferase III.

5 32. The oligonucleotide of claim 28, 29, 30 or 31 wherein the nucleic acid is DNA or RNA.

10 33. A nucleic acid having a sequence complementary to the sequence of the isolated nucleic acid of claim 1 or 2.

34. A method for determining whether a subject known to have an imbalance in sterol levels has the imbalance due to a defect in esterification of sterol which comprises:

15 (a) obtaining from the subject an appropriate sample containing a mixture of all of the subject's nucleic acids; and
(b) determining whether any nucleic acid in the sample from step (a) is, or is derived from, a
20 nucleic acid which encodes a mutant acylcoenzyme A: cholesterol acyltransferase so as to thereby determine whether the subject's imbalance in sterol levels is due to a defect in esterification of sterol.

25 35. The method of claim 34, wherein the determining of step (b) comprises:

(I) contacting the sample of step (a) with the isolated nucleic acid of claim 11 or 12 or
30 the oligonucleotide of claim 29 or 31 under conditions permitting binding of any nucleic acid in the sample which is, or is derived from, a nucleic acid which encodes a mutant acylcoenzyme A: cholesterol
35 acyltransferase to the nucleic acid or

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- oligonucleotide so as to form a complex;
- (ii) isolating the complex so formed; and
- (iii) identifying the nucleic acid in the isolated complex so as to thereby determine whether any nucleic acid in the sample contains a nucleic acid which is, or is derived from, a nucleic acid which encodes a mutant acylcoenzyme A: cholesterol acyltransferase II or III.

10

36. The method of claim 35, wherein the isolated nucleic acid or the oligonucleotide is labeled with a detectable marker.

15

37. The method of claim 36, wherein the detectable marker is a radioactive isotope, a fluorophore or an enzyme.

20

38. The method of claim 35, wherein the nucleic acid sample is first bound to a solid matrix before performing step (I).

25

39. The method of claim 35, wherein the sample comprises blood or sera.

30

40. A method for treating a subject who has an imbalance in sterol levels due to a defect in esterification of sterol which comprises introducing the isolated nucleic acid of claim 1 or 2 into the subject under conditions such that the nucleic acid expresses a wildtype acylcoenzyme A: cholesterol acyltransferase II or III, so as to thereby treat the subject.

35

41. A method for inhibiting wildtype acylcoenzyme A: cholesterol acyltransferase II or III in a subject



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which comprises transforming appropriate cells from the subject with a vector which expresses the nucleic acid of claim 33, and introducing the transformed cells into the subject so as to thereby
5 inhibit wildtype acylcoenzyme A: cholesterol acyltransferase II or III.

42. The method of claim 41, wherein the nucleic acid of claim 33 is capable of specifically hybridizing to
10 a mRNA molecule encoding acylcoenzyme A: cholesterol acyltransferase II or III so as to prevent translation of the mRNA molecule.

43. A method for inhibiting the wildtype acylcoenzyme A: cholesterol acyltransferase II or III in a subject
15 which comprises introducing the oligonucleotide of claim 28 or 30 into the subject so as to thereby inhibit the wildtype acylcoenzyme A: cholesterol acyltransferase II or III.

20 44. The method of claim 43, wherein the oligonucleotide of claim 28 or 30 is capable of specifically hybridizing to a mRNA molecule encoding acylcoenzyme A: cholesterol acyltransferase II or III so as to
25 prevent translation of the mRNA molecule.

45. A method for identifying a chemical compound which is capable of inhibiting acylcoenzyme A: cholesterol acyltransferase II or III in a subject which
30 comprises:

(a) contacting a wildtype acylcoenzyme A: cholesterol acyltransferase II or III with the chemical compound under conditions permitting binding between the acylcoenzyme and the
35 chemical compound;

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- (b) detecting specific binding of the chemical compound to the acylcoenzyme; and
- © determining whether the chemical compound inhibits the activity of the coenzyme so as to identify a chemical compound which is capable of inhibiting acylcoenzyme A: cholesterol acyltransferase II or III in a subject.
- 5
46. A pharmaceutical composition comprising the chemical compound identified by the method of claim 45 in an amount effective to inhibit acylcoenzyme A: cholesterol acyltransferase II or III in a subject and a pharmaceutically effective carrier.
- 10
47. A method of treating a subject who has atherosclerosis comprising administering the pharmaceutical composition of claim 46 to the subject.
- 15
48. A method of treating a subject who has hyperlipidemia comprising administering the pharmaceutical composition of claim 46 to the subject.
- 20
49. A transgenic, nonhuman mammal comprising the isolated nucleic acid of claim 1 or 2.
- 25

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FIG. 1A

```

1
ARE1 MTETKDLLQD EEFKIRRLN SAEANKRHSV TYDN..VILP QESMEVSPRS STTSLV.... EPVESTGV ESTEAEVAG KQEQEEYYPV DAIMQKYLSH
ARE2 MDKKKOLLN EQFLRIQKLN AADAGKQSI TVDDEGELYG LDTSGNSPAN EHTATTITQN HSWVASNGDV AFIPGTATEG NTEIVTTEVI ETDDNMFKTH
HACAT MVGEEKMSLR NRLSKSRENP EEDEDQNP. .... AKESL ETPSNGRIDI KOLIAKKIKL TAEAEEL...
CON M---KDLL-- E-FLKIR-LN -ADA-KR-S- T-D-----SP-- -T----- --V-S-E-V E-----R-G KQEI--E--- -A-----H

101
ARE1 LK...SKSRS RFIHKDABKY VSFFGDVSFD PRPTLLDSAI NVPFQTPKG PVLEKQL... ..TKTKAT VKTIVKTTEK
ARE2 VKT'LSSKEKA RY.KQGSBNF ISYFDDMSFE HRPSILDGVS NEPFKTKFVG PTLKEIRRR EKELMANHKN LHHRKSSPDA VDSVGKNDGA APTTVPTAAT
HACAT .....KP FFMKEVGBHF DDFVTNL.IE KSASLDNGGC AL...TTF.. SVLEGE.... KN NHRAKDLRAP PE.....Q GKIFIARRSL
CON -K---SK-K- RF-R---S-F -SFF-D-SFE -RPSLLDG-- N-PF-TTF-G PVLEKE---KN LH--K-----K-----K-ITV-T---

201
ARE1 TDKADAPPGE KLESNFSGIY VFAWMFLGWI AIRCCTDYA SYGSANWLE IVQYMTTDLF TIAMLDLAF LCTFFVVFVH WLKKRIINW KW'GFVAVSI
ARE2 SETVVTVEIT IISNFSGLY VAFWMAIAG AVKALIDYIY QHNGSFKDSE ILKFM'TTNLF TVASVDLLMY LSTYFVVGIQ YLCKWGLKW GTTGWIFTSI
HACAT LDELLEVD.. ....HIRTIV HMFIALLLIF ILSTLWVDYI DEGRVLLEFS LLSYAFGKFP TVVWTWIMF LSTFSVPYFL FQHWRTGYSK SSHPLIRSLF
CON -D---V--- ---SNFSGIY V-FWM-L--- A---L-DYY- --G-----E IL-YMTT-LF TVA--DL-MF LSTFFVW--- -L-K-----W --TG-I--SI

301
ARE1 FELAPIPVTF PIVVY..... ..YFDFNWVT RIFLFLHSV FVMKSHSPAF YNGYLWDIKQ ELEYSSKQLQ KYKESLS.PE TREILQKSCD FCLFELNYQT
ARE2 YEFLPVIFYM YLTEN..... ..ILKLHWLS KIFLFLHSLV LLMKMHSPAF YNGYLWGIKE ELQFSKSALA KYKDSINDPK VIGALEKSCE FCSFELSSQS
HACAT HGFLPMIFQI GVLGFGPTYV VLAYTLPPAS RFIIIFEQIR FVMKAHSP.. ....VRE NV...PRVLN SAKESSTVP IPTVNO.... ..
CON -EFLP-IF-- ----- -L-W-S RIFLFLHS-V FVMK-HSPAF YNGYLW-IKE EL--S---L- KYKES-S-P- ----LQKSC- FC-FEL--Q-

```

FIG. 1B

```

401
ARE1 KND...FPN NISCSNFFMP CLFPVLVYQI NYPRTSRIRW RYVLEKVCAL IGTFPLMMVT AQFFMHPVAM RCIQFHNTPT FGMIPATQE WFHLLFDMIP
ARE2 LSDQTKFPN NISAKSFFWP TMFTTLIYQI EYPRTKAIRW SYVLEKICAI FGTFPLMMID AQILMYPVAM RALAVRN.SE WTGILDRLLK WVGLLVDIVP
HACAT .....YLYP LFAPTLIYRD SYPRNPTVRW GYVAMKFAQV FQCFYVY...YIFERLCA PLFRNIKQEP FSARVLV...LCVFNSILP
CON -----FPN NIS-----FF-P --FPTLIYQI -YPRT--IRW -YVLEK-CAI FGTFPLMM--AQ--M-PVAM R-----N---F-G-----W--LL-DI-P
R07932 P

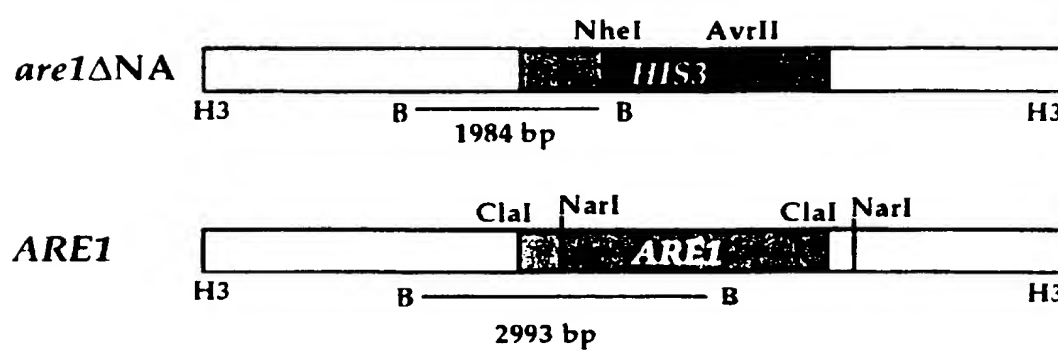
501
ARE1 GFTVLYMLTP YMIWDALLNC VABLTPFADR YFYGDMWNCV SFEEFSRIWN VPVHKFLLRH VYHSSMGAL. HLSKSQATLF .TFFLSAVVH EMAMFAIFRR
ARE2 GFIVMYILDY YLIWDAILNC VABLTPFGDR YFYGDMWNCV SWADFSRIWN IPVHKFLLRH VYHSSMSSF. KLNKSQATLM .TFFLSVSVH ELAMYVIFKK
HACAT GVLILF.LTP FAFLHCWLNA FABLTPFGDR MPYKDMWNST SYSNYRTWN VVHDWLYYY AYKDFLWFFS KRFXSAAML A .VFAVSAVVH EYALAVCLSF
CON GF-VLY-LTP Y-IWDA-LNC VABLTPFGDR YFYGDMWNCV S----FSRIWN VPVHKFLLRH VYHSSM--F- KL-KSQATL- .TFFLSAVVH E-AM-VIF--
R07932 NHLIWLIF.P YWLFHSCINA VABLTPFGDR EPYRDMWNCV SVTYFWQNWK IPVHKWCIRH FYKPMRLRGS ..SKWMARDR GVPGPSAFFH VVTW VSV P
** * * * * * ** * * * * * ** * * * * *

601
ARE1 VRGYLPMFOL SQF VWTALS NTKFLRARPO LSNVVSFGV CSQPSIIMTL YLTL.....
ARE2 LRFYLEFFQM LQ MPLVALT NTKFMRNRTI IGNVIFWLGI CMGPSVMCTL YLTF2.....
HACAT FYPVLPLVLFM FFGMAFNFIV NDS..RKKP. IWNVLMTSL FLONGVLLCF YSQEWYARRHC PLKNPTFLDY VRPRSWTCRY VF
CON -R-YLP-FQ- -Q-M---AL- NTKF-R-R- I-NV-FW-G- C-GPSV--TL YLT-----

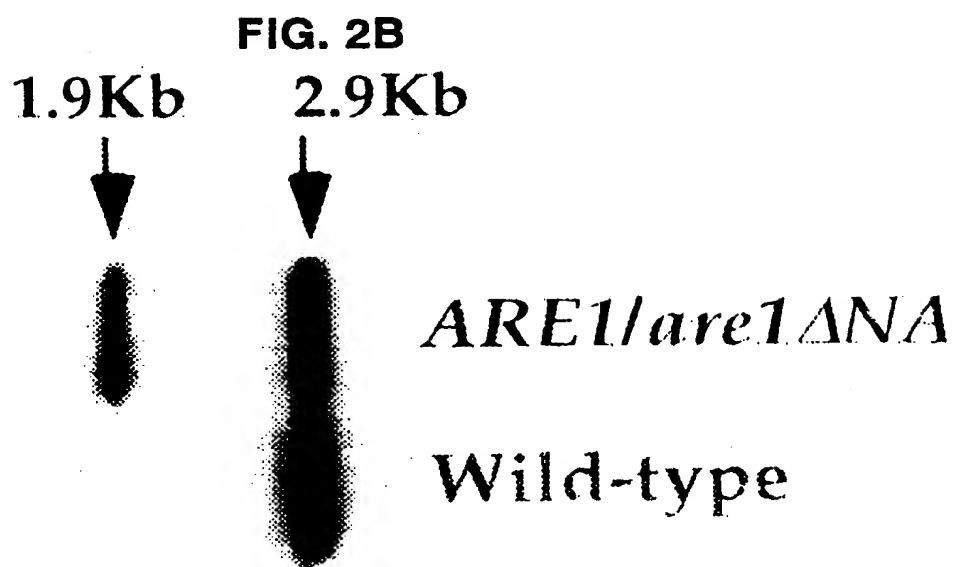
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FIG. 2A



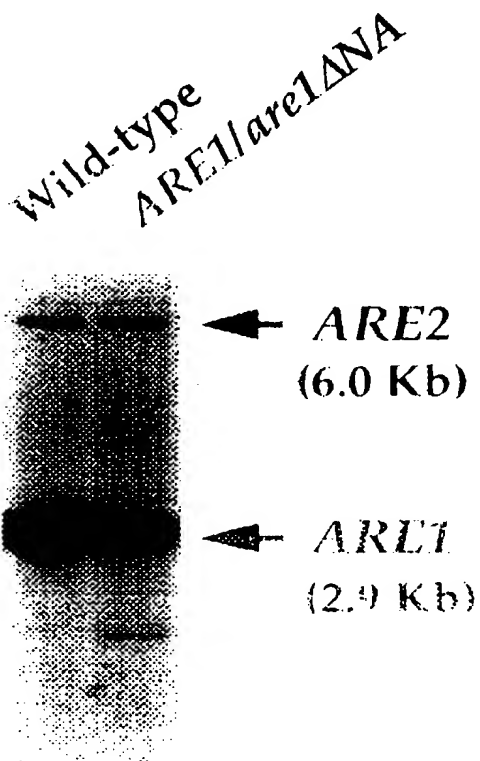
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SUBSTITUTE SHEET (RULE 26)

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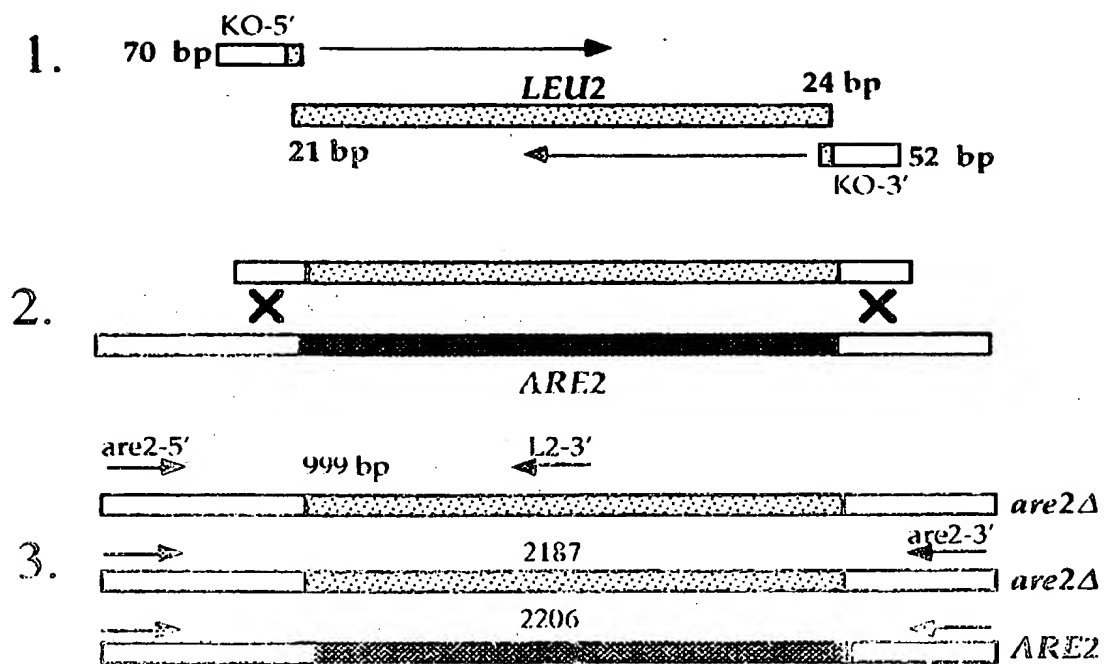
FIG. 2C



SUBSTITUTE SHEET (RULE 26)

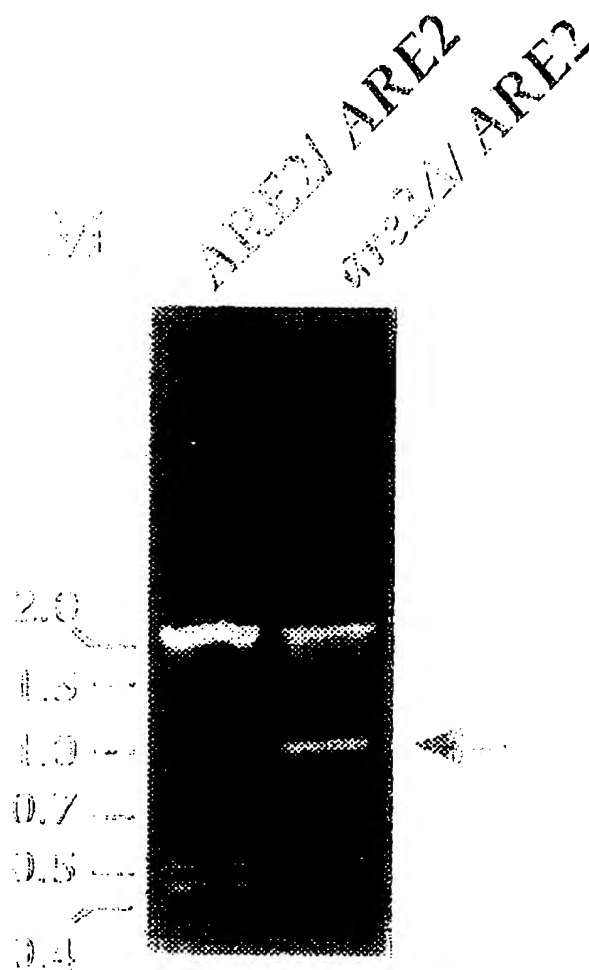
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FIG. 2D



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FIG. 2E



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FIG. 3A
Wild-type



FIG. 3B
are1 are2 mutant



SUBSTITUTE SHEET (RULE 26)

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FIG. 4B

Sterol Ester

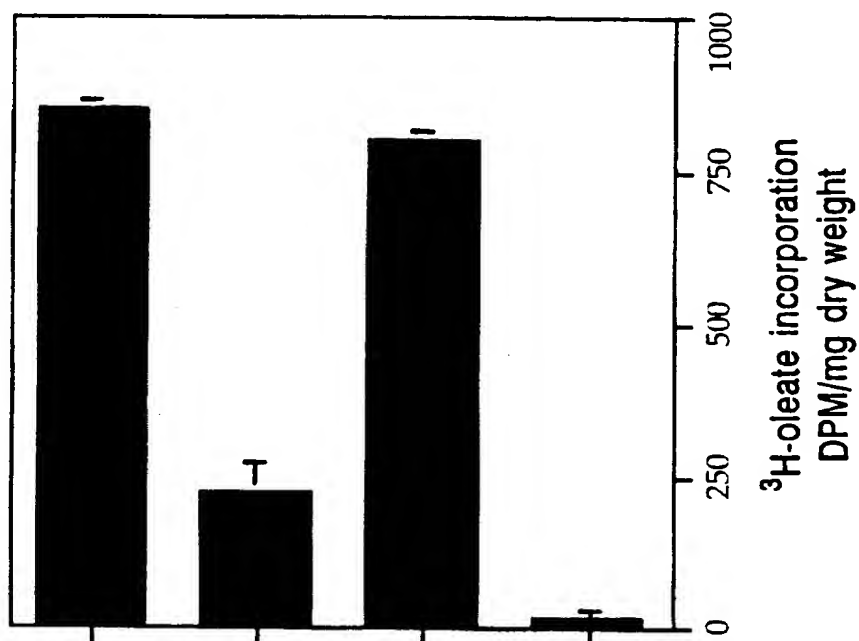
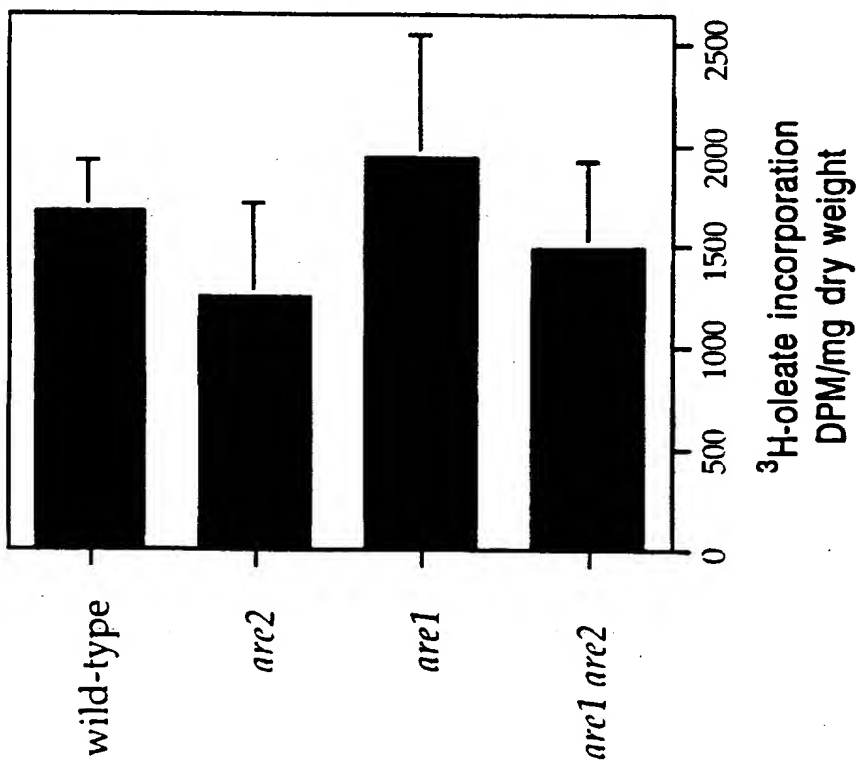


FIG. 4A

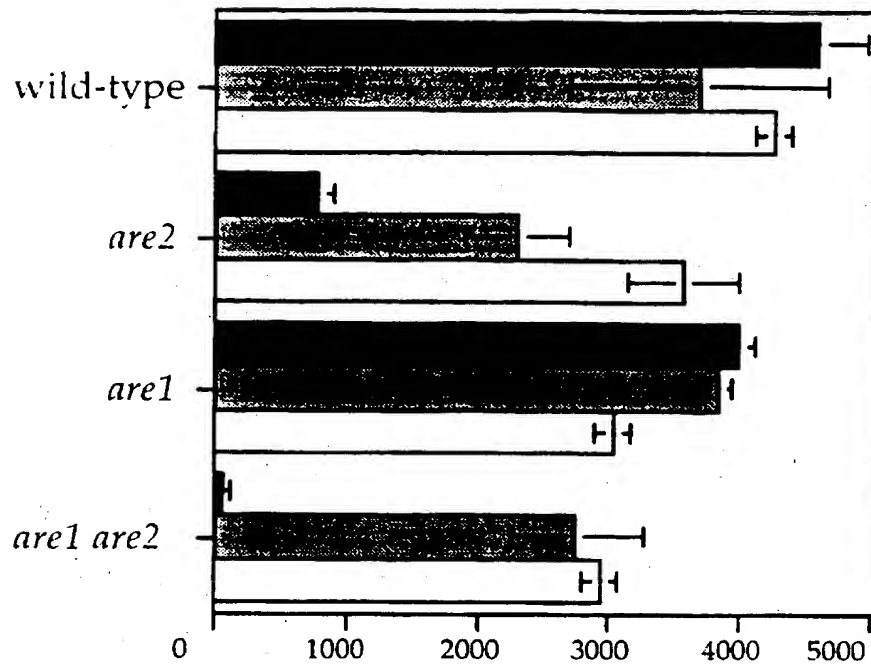
Triglyceride



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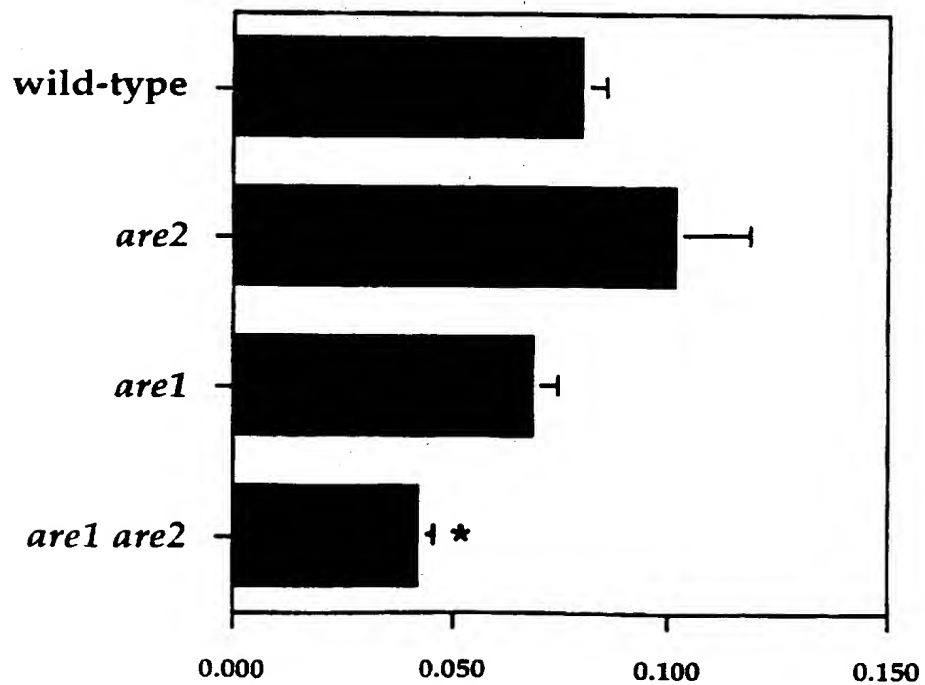
FIG. 4C

Sterol Ester



^3H -oleate incorporation
DPM/mg dry weight

FIG. 4D



^{14}C -acetate incorporation
(ratio of incorporation, sterol/fatty acid)

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FIG. 5A-1

```

1  gggtagagacggggttcaccgtgtagccaggatggtctggatctcctgacctgtagccaccacctcgccctcctaaagtctgggattacagaca 100
101  tgagccaccgcccagccctattcatccttttcaaaagttagaccctaggaagctggaggaggtgggcatggttttacagtgaatttctgatttca 200
201  ctgagggtgataaatcagactcttggggaagcgggtggtggctctggacagcagcaggaatggggatccagttagcaacaaatccatggacccatgacag 300
301  gctgaaagccacccttctccatcttgggaggttgccaatgtctgatttaacactatccaatgaatgatcattgaaagtataaaataaactatcaactag 400
401  cagaaaaatataaatggtaagcattagcacatatattcacatgtttatatattggctctcagattgacctataaaacaaagtctgggaaattctatatgatcc 500
501  tgaaaaaatgatacgtggtctggatggtagaataaagttggagaaatgtttaagccaaaatgcagctcttaccatgactttttattttattattatt 600
601  ttcaggatttttgggtatcacagggtgttttgggtacatggaaaagtcttctactggtgatttctgagatttttagttcacccctatccctgagcagtgta 700
701  actgttcccaatatgtagccttttatccctcacccctcctaagttcaagaagactatggtcctgcagaaaagctttatatgtaattaacatatctttatct 800
801  ttatctttataggcagtagactcatcttttgaacagattccattaaagtgatgaatgtgtacctcctcctctagcctttattattactgtttttgctattac 900
901  atgtgttagtgtgaatttaaatgttaaaatgtatcccatgtgctactatggcaaaaggttgactcataagagtttagcacgggttaagatctgaa 1000
1001  agttttctccagcctcttatcactggcgagacttcacaattcatggaaagccaccagtgagatgacattgcctcaggcagttactatttttatattctta 1100
1101  taactcgaggagctcagggttttcggaatcattaaacttttttgtcctttttaaagttggagacagcaattgtagacagccttcagtggttattctttt 1200
1201  tgtgtctccttacctglggagaagcctattagctgggatatgtagttaaatagctatatatttatatatatccagggcaccccggaattcgggagagcttccc 1300
1301  ggagtcgaccttctctgctgtgaccttcccggtctgacctctgcccgaagtgcctgctgcggggcgcggtcagacaataca  ATG 1399
1  1
1400  GTG GAT GAG AAG ATG TCT CTA AGA AAC CGG CTG TCA AAG TCC AGG GAA AAT CCT GAG GAA GAT GAA GAC CAG 1474
2  V G E K M S L R N R L S K S R E N P E D E D Q 26
1475  ACA AAC CCT GCA AAG GAG TCC CTA GAG ACA CCT AGT AGT AGT CGA ATT GAC ATA AAA CAG TTG ATA GCA AAG AAG 1549
27  R N P A K E S L E T P S N G R I D I K Q L I A K K 51
1550  ATA AAG TTG ACA GCA GAG GAA TTG AAG CCA TTT TTT ATG AAG GAA GTT GGC AGT CAC TTT GAT GAT TTT 1624
52  I K L T A E A E L K P F F M K E V G S H F D D F 76

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FIG. 5A-2

1625 GTG ACC AAT CTC ATT GAA AAG TCA GCA TCA TTA GAT AAT GGT GGG TGC GCT CTC ACA ACC TTT TCT GTT CTT GAA 1699
 77 V T N L I E K S A S L D N G G C A L T T F S V L E 101
 1700 GGA GAG AAA AAC CAT AGA GCG AAG GAT TTG AGA GCA CCT CCA GAA CAA GGA AAG ATT TTT ATT GCA AGG CGC 1774
 102 G E K N N H R A K D L R A P P E Q G K I F I A R R 126
 1775 TCT CTC TTA GAT GAA CTG CTT GAA CTG GAC CAC ATC AGA ACA ATA TAT CAC ATG TTT ATT GCC CTC CTC ATT CTC 1849
 127 S L L D E L L E V D H I R T I Y H M F I A L L I L 151
 1850 TTT ATC CTC AGC ACA CTT GTA GAT TAC TAC GAT AAT GAT GAA GGA AGG CTG GTG CTT GAG TTC AGC CTC CTG TCT TAT 1924
 152 F I L S T L V V D Y I D E G R L V L E F S L L S Y 176
 1925 GCT TTT GGC AAA TTT CCT ACC GTT GTT TGG ACC TGG TGG ATC ATG TTC CTG TCT ACA TTT TCA GTT CCC TAT TTT 1999
 177 A F G K F P T V V T W T W W I M F L S T F S V P Y F 201
 2000 CTG TTT CAA CAT TGG CGC ACT GGC TAT AGC AAG AGT TCT CAT CCG CTG ATC CGT TCT CTC TTC CAT GGC TTT CTT 2074
 202 L F Q H W R T G Y S K S S H P L I R S L F H G F L 226
 2075 TTC ATG ATC TTC CAG ATT GGA GTT CTA GGT TTT GGA CCA ACA TAT GTT GTG TTA GCA TAT ACA CTG CCA CCA GCT 2149
 227 F M I F Q I G V L G F G P T Y V V L A Y T L P P A 251
 2150 TCC CGG TTC ATC ATT ATA TTC GAG CAG ATT CGT TTT GTA ATG AAG GCC CAC TCA TTT GTC AGA GAG AAC GTG CCT 2224
 252 S R F I I I F E Q I R F V M K A H S F V R E N V P 276
 2225 CGG GTA CTA AAT TCA GCT AAG GAG AAA TCA AGC ACT GTT CCA ATA CCT ACA GTC AAC CAG TAT TTG TAC TTC TTA 2299
 277 R V L N S A K E K S S T V P I P T V N Q Y L Y F L 301
 2300 TTT GCT CCT ACC CTT ATC TAC CGT GAC AGC TAT CCC AGG AAT CCC ACT GTA AGA TGG GGT TAT GTC GCT ATG AAG 2374
 302 F A P T L I Y R D S Y P R N P T V R W G Y V A M K 326
 2375 TTT GCA CAG GTC TTT GGT TGC TTT TTC TAT GTG TAC TAC ATC TTT GAA AGG CTT TGT GCC CCC TTG TTT CGG AAT 2449
 327 F A Q V F G C F F Y V Y I F E R L C A P L F R N 351
 2450 ATC AAA CAG GAG CCC TTC AGC GCT CGT GTT CTG GTC CTA TGT GTA TTT AAC TCC ATC TTG CCA GGT GTG CTG ATT 2524
 352 I K Q E P F S A R V L V L C V F N S I L P G V L I 376

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FIG. 5A-3

2525 CTC TTC CTT ACT TTT TTT GCC TTT TTG CAC TGC TGG CTC AAT GCC TTT GCT GAG ATG TTA CGC TTT GGT GAC AGG 2599
 377 L F L T F A F L L H C W L N A F A E M L R F G D R 401
 2600 ATG TTC TAT AAG GAT TGG TGG AAC TCC ACG TCA TAC TCC AAC TAT TAT AGA ACC TGG AAT GTG GTG GTC CAT GAC 2674
 402 M F Y K D W N S T S Y S N Y Y Y R T W N V V V H D 426
 2675 TGG CTA TAT TAC TAT GCT TAC AAG GAC TTT CTC TGG TTT TTC TCC AAG AGA TTC AAA TCT GCT GCC ATG TTA GCT 2749
 427 W L Y Y A Y K D F L W F F S K R F K S A A M L A 451
 2750 GTC TTT GCT GTA TCT GCT GTA CAC GAA TAT GCC TTG GCT GTT TGC TTG AGC TTT TTC TAT CCC GTG CTG TTC 2824
 452 V F A V S A V V H E Y A L A V C L S F F Y P V L F 476
 2825 GTG CTC TTC ATG TTC TTT GGA ATG GCT TTC AAC TTC ATT GTC AAT GAT AGT CCG AAA AAG CCG ATT TGG AAT GTT 2899
 477 V L F M F F G M A F N F I V N D S R K K P I W N V 501
 2900 CTG ATG TGG ACT TCT CTT TTC TTT GGC AAT GGA GTC TTA CTC TGC TTT TAT TCT CAA GAA TGG TAT GCA CGT CGG 2974
 502 L M W T S L F L G N G V L L C F Y S Q E W Y A R R 526
 2975 CAC TGT CCT CTG AAA AAT CCC ACA TTT TTG GAT TAT GTC CGG CCA CGT TCC TGG ACT TGT CGT TAC GTC TTT TAG 3049
 527 H C P L K N P T F L D Y V R P R S W T C R Y V F * 551
 3050 aagcttggaactttgttccctctgactgaagattgggttagctccctgatttggagccagctgtttccagttgttactgaagttatctgtgtatttg 3149
 3150 gaccactccaggctttacagatgactcactccattccttaggtcacttgaagccaaactgttgaagttcactggagttctgtacacttaagcagagcaga 3249
 3250 actttttttgtgggctgggtggggggaagaccgactaacagctgaagtaatgacagattgttgcgtgggtcataatcagctttatcccttgtaattat 3349
 3350 atctgtttttgttcttgactctgtccaatcagagaataaacatcatagtttcttggccactgaattagccaaaacacttaggaagaatacacttaaatatc 3449
 3450 ctctggcttagaaaattttttcatgcacactgttgggaatgtatgctaattgaacatgcaattggggaagaaaaaatgtagaatgatttttgcatttctag 3549
 3550 tagaaagaaaatgtctgttttccaaaagataatgtttatacatcctatttttgaatttttttgaaaaaaagtttcaatgttcagttttcccttagttttacctt 3649

FIG. 5B-1

1 gccctccagctcttactaagaccggtcggaagcatgctggcgatatataccaaaccacacacacatggctcctcctcgctgctcaaaaatctccccagac 200

101 agtcgggacccgcaccggatccagaatgaaactgcacggctgcagattcaaaagctccaacggcctcagcgtcatcttcgctcggtatgctgcactc 200

201 tggctgaaccgctgactgtgtgcttcgtatcattatagaaaatctcgggtggcgcaactcctcaggacgtgacattattcttctctgatattt 300

301 cctgtgtttccgtaaccgcacctttttagcactacttttttactatgctcttcttctctgcttcttctctgttttttcttcttccactatgtatgtg 400

401 ctgctcatctctcttttatcgataaaaattgaaaatgtgagatgggtgagagtgaiaaaaaaataatctggttggccatcaaataccggccgt 500

501 ggltggactcgttttagcgaacaatagcaccacccagcagccctggcaacatgcggatgatataagaaggacgagcgtggtgaggaaaagggcgccattggc 600

601 acactcacgcaggtggtgttgcagcacggttgcagcaagcgcgaacacagattgcaaga ATG ACG GAG ACT AAG GAT TTG TTG CAA 689
1 M T E T K D L L Q 9

690 GAC GAA GAG TTT CTT AAG ATC CGC AGA CTC AAT TCC GCA GAA GCC AAC AAA CGG CAT TCG GTC ACG TAC GAT AAC 764
10 D E E F L K I R L N S A E A N K R H S V T Y D N 34

765 GTG ATC CTG CCA CAG GAG TCC ATG GAG TTC TCG CCA CGG TCG TCT ACC ACG TCG CTG GTG GAG CCA GTG GAG TCG 839
35 V I L P Q E S M E V S P R S S T T S L V E P V E S 59

840 ACT GAA CGA GTG GAG TCG ACT GAG GCG GAA CGT GTG GCA GGG AAG CAG GAG CAG GAG GAG TAC OCT GTG GAC 914
60 T E G V E S T E A E R V A G K Q E E E Y P V D 84

915 GCC CAC ATG CAA AAG TAC CTT TCA CAC CTG AAG AGC AAG TCT CGG TCG AGG TTC CAC CGA AAG GAT GCT AGC AAG 989
85 A H M Q K Y L S H L S K S R S R F H R K D A S K 109

990 TAT GTG TCG TTT TTT GGG GAC GTG AGT TTT GAT CCT CGC CCC ACG CTC CTG GAC AGC GCC ATC AAC GTG CCC TTC 1064
110 Y V S F F G D V S F D P R P T L L D S A I N V P F 134

1065 CAG ACG ACT TTC AAA CGT CCG GTG CTG GAG AAA CAG CTC AAA AAT TTA CAG TTG ACA AAG ACC AAG ACC AAG GCC 1139
135 Q T T F K G P V L E K Q L K N L Q L T K T K T K A 159

1140 ACG GTG AAG ACT ACG GTG AAG ACT ACG GAG AAA ACG GAC AAG GCA GAT GCC CCC CCA GGA GAA AAA CTG GAG TCG 1214
160 T V K T T V K T T E K T D K A D A P P G E K L E S 184

1215 AAC TTT TCA GGG ATC TAC GTG TTC GCA TGG ATG TTC TTG GGC TGG ATA GCC ATC AGG TGC TGC ACA GAT TAC TAT 1289
185 N F S G I Y V F A W M F L G W I A I R C C T D Y Y 209

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FIG. 5B-2

1290 GCG TCG TAC GGC AGT GCA TGG AAT AAG CTG GAA ATC GTG CAG TAC ATG ACA ACG GAC TTG TTC ACG ATC GCA ATG 1364
 210 A S Y G S A W N K L E I V Q Y M T T D L F T I A M 234
 1365 TTG GAC TTG GCA ATG TTC CTG TGC ACT TTC TTG GTG GTT TTC GTG CAC TGG CTG GTG AAA AAG CGG ATC ATC AAC 1439
 235 L D L A M F L C T F F V V F V H W L V K K R I I N 259
 1440 TGG AAG TGG ACT GCG TTC GTT GCA GTG AGC ATC TTC GAG TTG GCT TTC ATC CCC GTG ACG TTC CCC ATT TAC GTC 1514
 260 W K W T G F V A V S I F E L A F I P V T F P I Y V 284
 1515 TAC TAC TTT GAT TTC AAC TGG GTC ACG AGA ATC TTC CTG TTC CTG CAC TCC GTG GTG TTT GTT ATG AAG AGC CAC 1589
 285 Y Y F D F N W V T R I F L F L H S V V F V M K S H 309
 1590 TCG TTT GCC TTT TAC AAC GGG TAT CTT TGG GAC ATA AAG CAG GAA CTC GAG TAC TCT TCC AAA CAG TTG CAA AAA 1664
 310 S F A F Y N G Y L W D I K Q E L E Y S S K Q L Q K 334
 1665 TAC AAG GAA TCT TTG TCC CCA GAG ACC CGC GAG ATT CTG CAA AAA AGT TGC GAC TTT TGC CTT TTC GAA TTG AAC 1739
 335 Y K E S L S P E T R E I L Q K S C D F C L F E L N 359
 1740 TAC CAG ACC AAG GAT AAC GAC TTC CCC AAC ATC ATC AGT TGC AGC AAT TTC ATG TTC TGT TGT TTC CCC GTC 1814
 360 Y Q T K D N D F P N N I S C S N F F M F C L F P V 384
 1815 CTC GTG TAC CAG ATC AAC TAC CCA AGA ACG TCG CGC ATC AGA TGG AGG TAT GTG TTG GAG AAG GTG TGC GCC ATC 1889
 385 L V Y Q I N Y P R T S R I R W R Y V L E K V C A I 409
 1890 ATT GGC ACC ATC TTC CTC ATG ATG GTC ACG GCA CAG TTC TTG CAC CCG GTG GCC ATG CGC TGT ATC CAG TTC 1964
 410 I G T I F L M M V T A Q F F M H P V A M R C I Q F 434
 1965 CAC AAC ACG ACC TTC GGC GGC TGG ATC CCC GCG ACG CAA GAG TGG TTC CAC CTG CTC TTC GAC ATG ATT CCG 2039
 435 H N T P T F G G G W I P A T Q E W F H L L F D M I P 459
 2040 GGC TTC ACT GTT CTG TAC ATG CTC ACG TTT TAC ATG ATA TGG GAC GCT TTA TTG AAT TGC GTG GCG GAG TTG ACC 2114
 460 G F T V L Y M L T F Y M I W D A L L N C V A E L T 484

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FIG. 5B-3

2115 AGG TTT GCG GAC AGA TAT TTC TAC GAC TGG TGG AAT TGC GTT TCG TTT GAA GAG TTT AGC AGA ATC TGG AAC 2189
 485 R F A D R Y F Y G D W W N C V S F E E F S R I W N 509
 2190 GTC CCC GTT CAC AAA TTT TTA CTA AGA CAC GTG TAC CAC AGC TCC ATG GGC GCA TTG CAT TTG AGC AAG AGC CAA 2264
 510 V P V H K F L L R H V Y H S S M G A L H L S K S Q 534
 2265 GCT ACA TTA TTT ACT TTT TTC TTG AGT GCC GTG TTC CAC GAA ATG GCC ATG TTC GCC ATT TTC AGA AGG GTT AGA 2339
 535 A T L F T F F L S A V F H E M A M F A I F R R V R 559
 2340 GGA TAT CTG TTC ATG TTC CAG TCG CAG TTT GTG TGG ACT GCT TTG AGC AAC ACC AAG TTT CTA CCG GCA AGA 2414
 560 G Y L F M F Q L S Q F V W T A L S N T K F L R A R 584
 2415 CCG CAG TTG TCC AAC GTT GTC TTT TCG TTT GGT GTC TGT TCA GGG CCC AGT ATC ATT ATG ACG TTG TAC CTG ACC 2489
 585 P Q L S N V V F S F G V C S G P S I I M T L Y L T 609
 2490 TTA TGA actgcccaccataccacgtgtgtccctcgcaagcccttgatagatatacaatagggaatgggcgtccgtccaccgtggtcacaagacaggggca 2587
 610 L * 611
 2588 aagagctcctaggt 2601

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FIG. 5C-1

1 tataaaattccttcatcaatacatctatatattcgaatatatagataaaccatacaaaaacatactgaaattttttgaaaacaaactaaaaactattcat 100
 101 tgcagttacacgtgaatgctaaactttatatcgtctcttgctgccccgaggttaacatttaacggcttctcgcgcaataaccggaataattccaacag 200
 201 tttcttttgtaataattattaagccttctttttcccggaatctataagaggggacgaaaattagccgctatttaattctggtattgccacctagacaagaag 300
 301 taaacagacacattacgttagcaaaagcaacaatacaacaacaacc ATG GAC AAG AAG AAG GAT CTA CTG GAG AAC GAA CAA TTT 386
 1 M D K K K D L L E N E Q F 13
 387 CTC CGC ATC CAA AAG CTC AAC GCT GCC GAT GCG GGC AAA AGA CAA TCT ATA ACA GTG GAC GAC GAG GGC GAA CTA 461
 14 L R I Q K L N A A D A G K R Q S I T V D D E G E L 38
 462 TAT GGG TTA GAC ACC TCC GGC AAC TCA CCA GCC AAT GAA CAC ACA GCT ACC ACA ATT ACA CAG AAT CAC AGC GTG 536
 39 Y G L D T S G N S P A N E H T A T T I T Q N H S V 63
 537 GTG GCC TCA AAC GGA GAC GTC GCA TTC ATC CCA GGA ACT GCT ACC GAA GGC AAT ACA GAG ATT GTA ACT GAA GAA 611
 64 V A S N G D V A F I P G T A T E G N T E I V T E E 88
 612 GTG ATT GAG ACC GAT AAC ATG TTC AAG ACC CAT GTG AAG ACT TTA AGC TCC AAA GAG AAG GCA CGG TAT AGG 686
 89 V I E T D D N M F K T H V K T L S S K E K A R Y R 113
 687 CAA GGG TCC TCT AAC TTT ATA TCG TAT TTC GAT GAT ATG TCA TTT GAA CAC AGG CCC AGT ATA TTA GAT GCG TCA 761
 114 Q G S S N F I S Y F D D M S F E H R P S I L D G S 138
 762 GTT AAC GAG CCC TTC AAG ACC AAA TTC GTG GGA CCT ACT TTA GAA AAG GAG ATC AGA AGG GAG AAA GAG CTA 836
 139 V N E P F K T K F V G P T L E K E I R R E K E L 163
 837 ATG GCC ATG CGC AAA AAT TTA CAC CAC CGC AAG TCC TCC CCA GAT GCT GTC GAC TCA GTA GGG AAA AAT GAT GGC 911
 164 M A M R K N L H H R K S S P D A V D S V G K N D G 188
 912 GCC GCC CCA ACT ACT GTT CCA ACT GCC ACC TCA GAA ACG GTG GTC ACC GTT GAA ACC ACC ATA ATT TCA TCC 986
 189 A A P T T V P T A A T S E T V V T V E T T I I S S 213
 987 AAT TTC TCC GGG TTG TAC GTG GCG TTT TGG ATG GCT ATT GCA TTT GGT GCT GTC AAG GCT TTA ATA GAC TAT TAT 1061
 214 N F S G L Y V A F W M A I A F G A V K A L I D Y Y 238

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FIG. 5C-2

1062	TAC	CAG	CAT	AAT	GGT	AGC	ATC	TTC	AAG	GAT	TCG	GAG	ATC	TTT	AAA	TTT	ATG	ACT	ACG	AAT	TTG	TTC	ACT	GTG	GCA	TCC	1136
239	Y	Q	H	N	G	S	F	K	D	S	E	I	L	K	F	M	T	N	L	F	T	V	A	S		263	
1137	GTA	GAT	CTT	TTG	ATG	TAT	TTT	GTC	GTT	GGA	ATA	CAA	TAC	TTA	TGC	AAG	TCG	GGG	GTC	TTG	AAA					1211	
264	V	D	L	L	M	Y	L	S	T	Y	F	V	G	I	Q	Y	L	C	K	W	G	V	L	K		288	
1212	TGG	GGC	ACT	ACC	GGC	TGG	ATC	TTC	ACC	TCA	ATT	TAC	GAG	TTT	TTG	TTT	GTT	ATC	TTC	TAC	ATG	TAT	TTA	ACA	GAA	1286	
289	W	G	T	T	G	W	I	F	T	S	I	Y	E	F	L	F	V	I	F	Y	M	Y	L	T	E	313	
1287	AAC	ATC	CTA	AAA	CAC	TGG	CTG	TCC	AAG	ATC	TTC	CTT	TTT	TTG	CAT	TCT	TTA	GTT	TTA	TTG	ATG	AAA	ATG	CAT		1361	
314	N	I	L	K	L	H	W	L	S	K	I	F	L	F	L	H	S	L	V	L	L	M	K	M	H	338	
1362	TCT	TTC	GCC	TTC	TAC	AAT	GGC	TAT	CTA	TGG	GGT	ATA	AAG	GAA	GAA	CTA	CAA	TTT	TCC	AAA	AGC	GCT	CTT	GCC	AAA	1436	
339	S	F	A	F	Y	N	G	Y	L	W	G	I	K	E	E	L	Q	F	S	K	S	A	L	A	K	363	
1437	TAC	AAG	GAT	TCT	ATA	AAT	GAT	CCA	AAA	GTT	ATT	GGT	GCT	CTT	GAG	AAA	AGC	TGT	GAG	TTT	TGT	AGT	TTT	GAA	TTG	1511	
364	Y	K	D	S	I	N	D	P	K	V	I	G	A	L	E	K	S	C	E	F	C	S	F	E	L	388	
1512	AGC	TCT	CAG	TCT	TTA	AGC	GAC	CAA	ACT	CAA	AAA	TTC	CCC	AAC	AAT	ATC	AGT	GCA	AAA	AGC	TTT	TTT	TGG	TTC	ACC	1586	
389	S	S	Q	S	L	S	D	Q	T	Q	K	F	P	N	N	I	S	A	K	S	F	F	W	F	T	413	
1587	ATG	TTT	CCA	ACC	CTA	ATT	TAC	CAA	ATT	GAA	ATT	CCA	AGA	ACT	AAG	GAA	ATC	AGA	TGG	AGC	TAC	GTA	TTA	GAA	AAG	1661	
414	M	F	P	T	L	I	Y	Q	I	E	Y	P	R	T	K	E	I	R	W	S	Y	V	L	E	K	438	
1662	ATC	TGC	GCC	ATC	TTC	GGT	ACC	ATT	TTC	TTA	ATG	ATA	GAT	GCT	CAA	ATC	TTG	ATG	TAT	CCT	GTA	GCA	ATG	AGA		1736	
439	I	C	A	I	F	G	T	I	F	L	M	M	I	D	A	Q	I	L	M	Y	P	V	A	M	R	463	
1737	GCA	TTG	GCT	GTG	CGC	AAT	TCT	GAA	TGG	ACT	ATA	TTG	GAT	AGA	TTA	TTG	AAA	TGG	GTT	GGA	TTG	CTC	GTT	GAT		1811	
464	A	L	A	V	R	N	S	E	W	T	G	I	L	D	R	L	L	K	W	V	G	L	L	V	D	488	
1812	ATC	GTC	CCA	GGG	TTT	ATC	GTG	ATG	TAC	ATC	TTG	GAC	TTC	TAT	TTG	ATT	TGG	GAT	GCC	ATT	TTG	AAC	TGT	GTG	GCT	1886	
489	I	V	P	G	F	I	V	M	Y	I	L	D	F	Y	L	I	W	D	A	I	L	N	C	V	A	513	
1887	GAA	TTG	ACA	AGA	TTT	GGC	GAC	AGA	TAT	TTT	TAC	GGT	GAC	TGG	TGG	AAT	TGT	GTT	AGT	TGG	GCA	GAC	TTC	AGT	AGA	1961	
514	E	L	T	R	F	G	D	R	Y	F	Y	G	D	W	W	N	C	V	S	W	A	D	F	S	R	538	

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FIG. 5C-3

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1962 ATT TGG AAC ATC CCA GTG CAT AAG TTT TTG TTA AGA CAT GTT TAC CAT AGT TCA ATG AGT TCA TTC AAA TTG AAC 2036
539 I W N I P V H K F L L R H V Y H S S M S S F K L N 563

2037 AAG AGT CAA GCA ACT TTG ATG ACC TTT TTC TTA AGT TCC GTC GTT CAT GAA TTA GCA ATG TAC GTT ATC TTC AAG 2111
564 K S Q A T L M T F F L S S V V H E L A M Y V I F K 588

2112 AAA TTG AGG TTT TAC TTG TTC TTC CAA ATG CTG CAA ATG CCA TTA GTA GCT TTA ACA AAT ACT AAA TTC ATG 2186
589 K L R F Y L F F Q M L Q M P L V A L T N T K F M 613

2187 AGG AAC AGA ACC ATA ATC GGA AAT GTT ATT TTC TGG CTC GGT ATC TGC ATG GGA CCA AGT GTC ATG TGT ACG TTG 2261
614 R N R T I I G N V I F W L G I C M G P S V M C T L 638

2262 TAC TTG ACA TTC TAA * ggcacccctgcaactgttctgtggagctattaaatcttttatagtaaaattttttttacttttttttttttttttttttt 2356
639 Y L T F 643

2357 tttattattacaagcgctctatatattttctattatagaatatattgtcattttattacattggttca 2421

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FIG. 5D

1	ATG	GAC	AAC	GCG	TCT	GAT	ACG	ACT	CAC	TAT	AGG	GAA	TTT	GGC	CCT	CGA	GCA	GTA	GAT	TCG	GCA	CGA	TGG	GCA	75	
1	M	D	N	A	G	S	D	T	H	Y	R	E	F	G	P	R	A	V	D	S	A	R	W	A	25	
76	CGA	GGA	CTC	CAT	GTT	CCT	CAA	GCT	TTA	TTC	CTA	CCG	GGA	TGT	CAA	CCT	GTG	GTG	CCG	CCA	GCG	AAG	GGT	CAA	150	
26	R	G	L	H	V	P	Q	A	L	F	L	P	G	C	Q	P	V	V	P	P	A	K	G	Q	50	
151	GGC	CAA	ACC	TCT	CTC	TAC	AGG	GAA	GAA	GGT	CAG	TGG	GGC	TGC	TGC	GAG	CAA	GCT	GTG	AGC	TAT	CCA	GAC	AAC	CTG	225
51	G	Q	S	C	L	Y	R	E	E	G	Q	W	G	C	C	E	Q	A	V	S	Y	P	D	N	L	75
226	ACC	TAC	CGA	GAT	CTC	GAT	TAC	TTC	ATC	TTT	GCT	CCT	ACT	TTG	TGT	TAT	GAA	CTC	AAC	TTT	CCT	CGG	TCC	CCC	CGA	300
76	T	Y	R	D	L	D	Y	F	I	F	A	P	T	L	C	Y	E	L	N	F	P	R	S	P	R	100
301	ATA	CGA	GAG	CGC	TTT	CTG	CTA	CGA	GTT	CTT	GAG	ATG	CTC	TTT	TTT	ACC	CAG	CTT	CAA	GTG	GCG	CTG	ATC	CAA	375	
101	I	R	E	R	F	L	L	R	R	V	L	E	M	L	F	T	Q	L	Q	V	G	L	I	Q	125	
376	CAG	TGG	ATG	GTC	CCT	ACT	ATC	CAG	AAC	TCC	ATG	GAA	GCC	CTT	TCA	AGA	GCT	TCT	GGC	AGT	TTT	GGA	GAC	CGC	GAG	450
126	Q	W	M	V	P	T	I	Q	N	S	M	E	A	L	S	R	A	S	G	S	F	G	D	R	E	150
451	TTT	TAC	AGA	GAT	TGG	TGG	AAT	GCT	GAG	TCT	GTC	ACC	GAC	TTT	TGG	CAG	AAC	TGG	AAT	ATC	CCC	GTG	G		517	
151	F	Y	R	D	W	W	N	A	E	S	V	T	D	F	W	Q	N	W	N	I	P	V			172	

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FIG. 6A

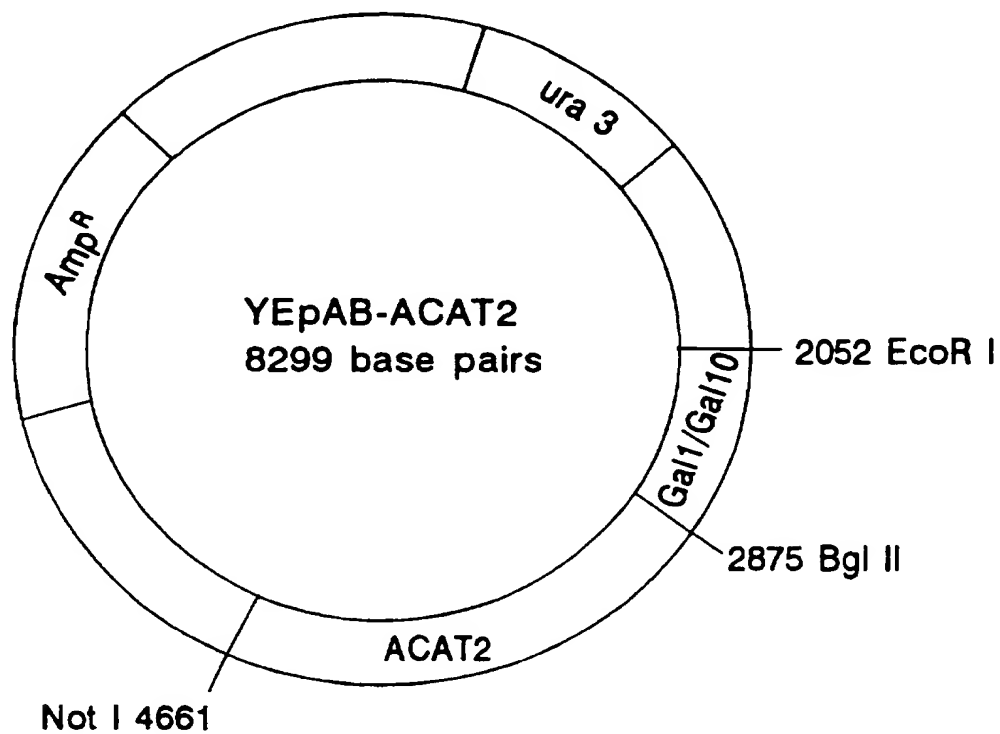
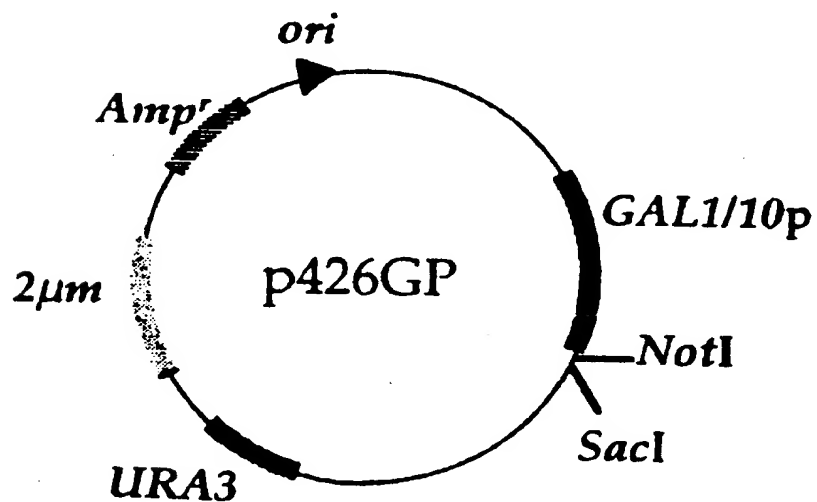
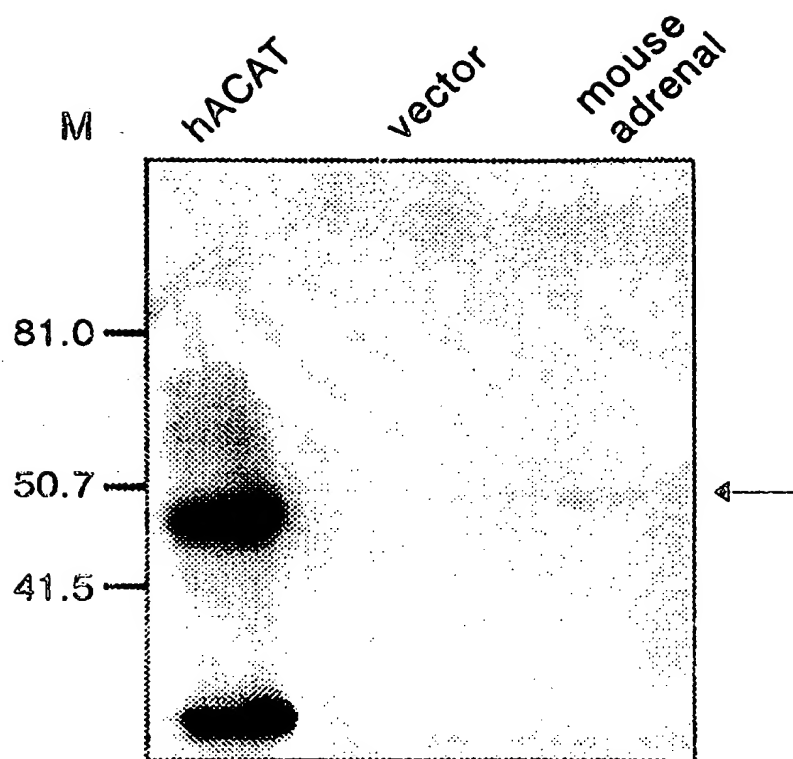


FIG. 6B



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FIG. 6C

 α hACAT, DM10

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FIG. 7B

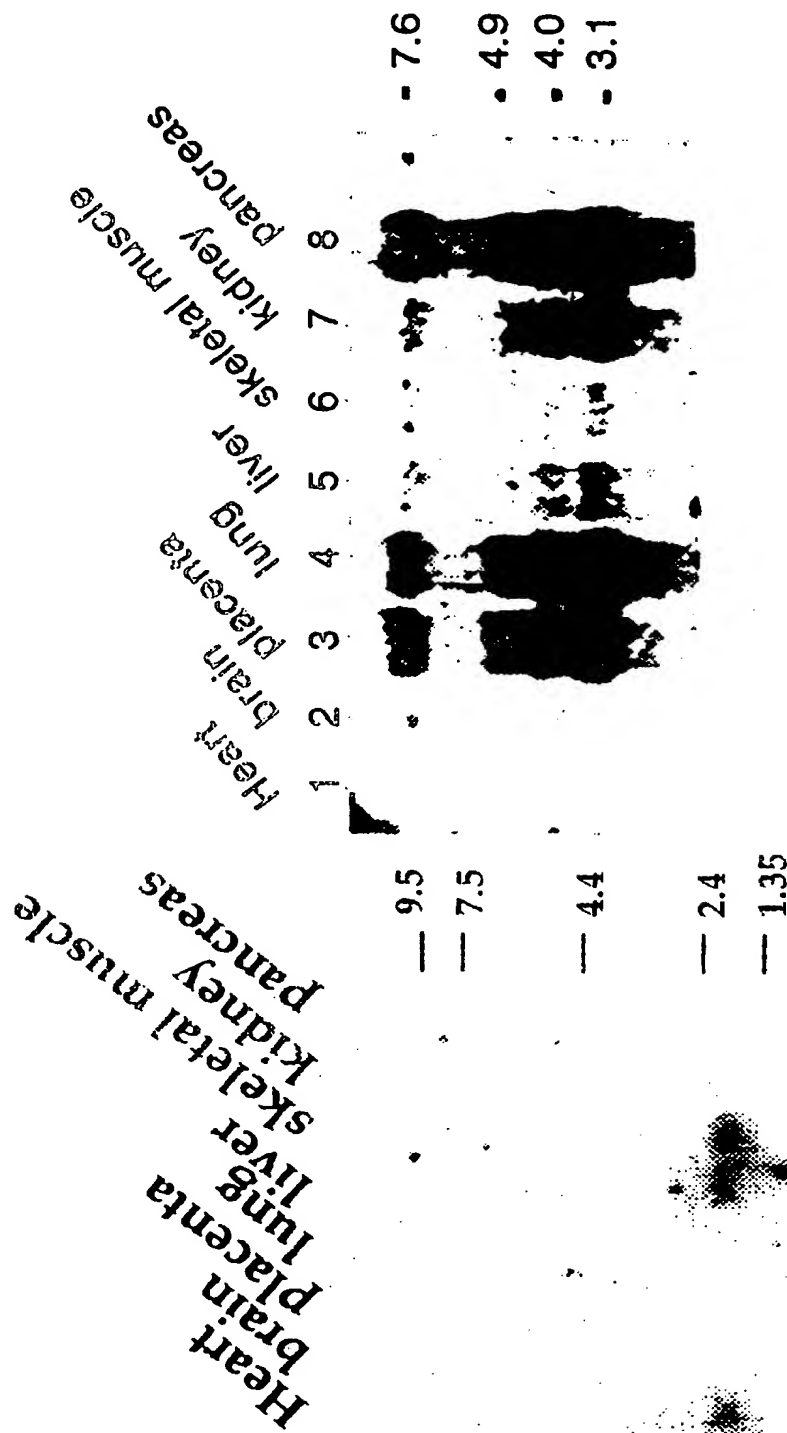


FIG. 7A

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FIG. 8A

FIG. 8B

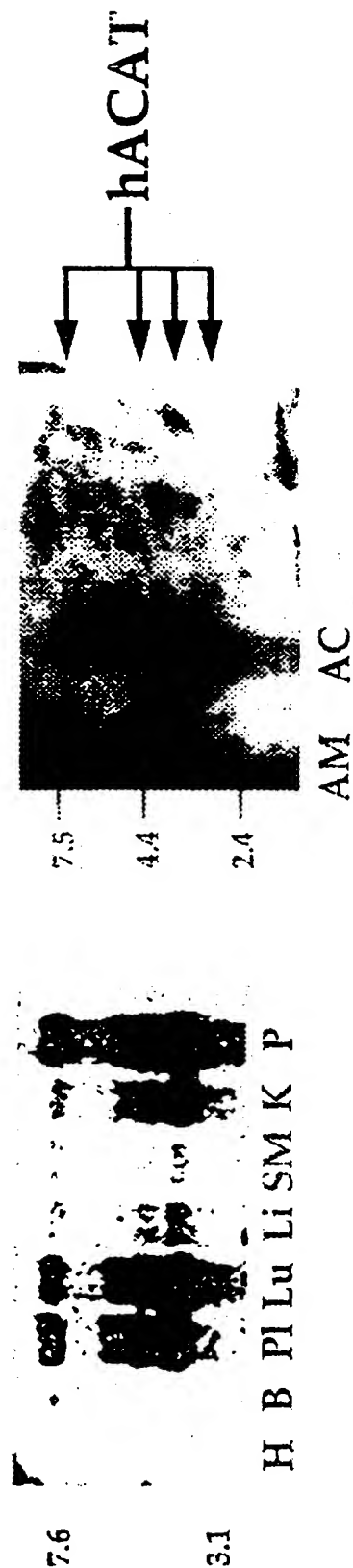
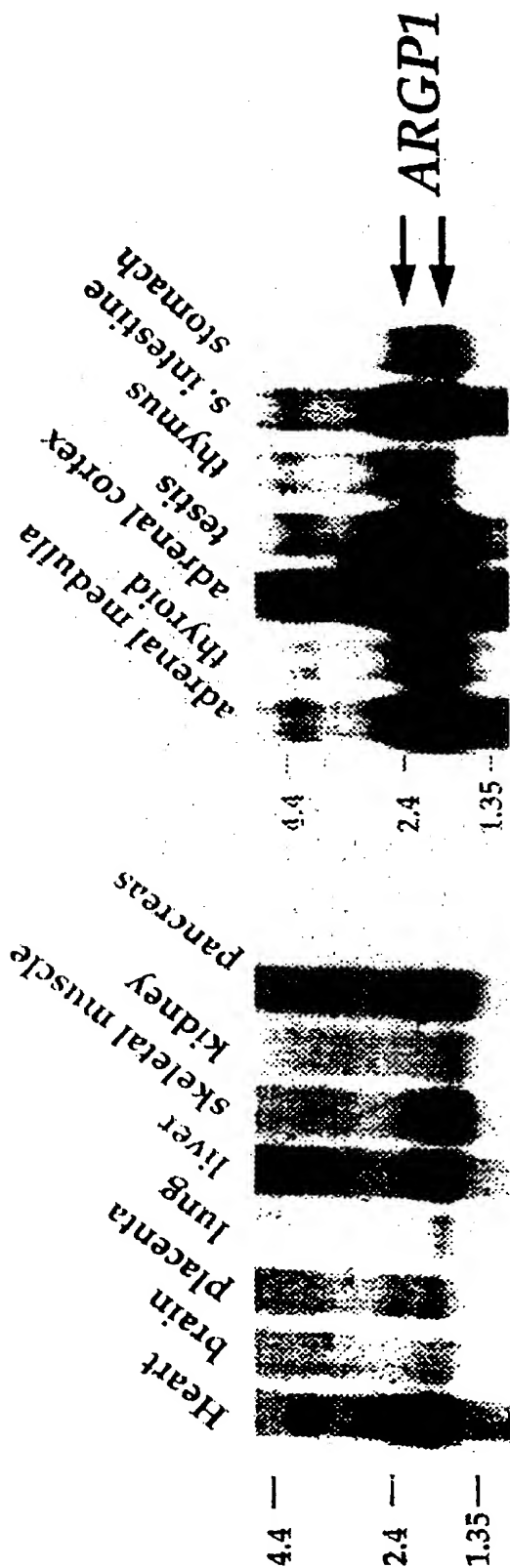
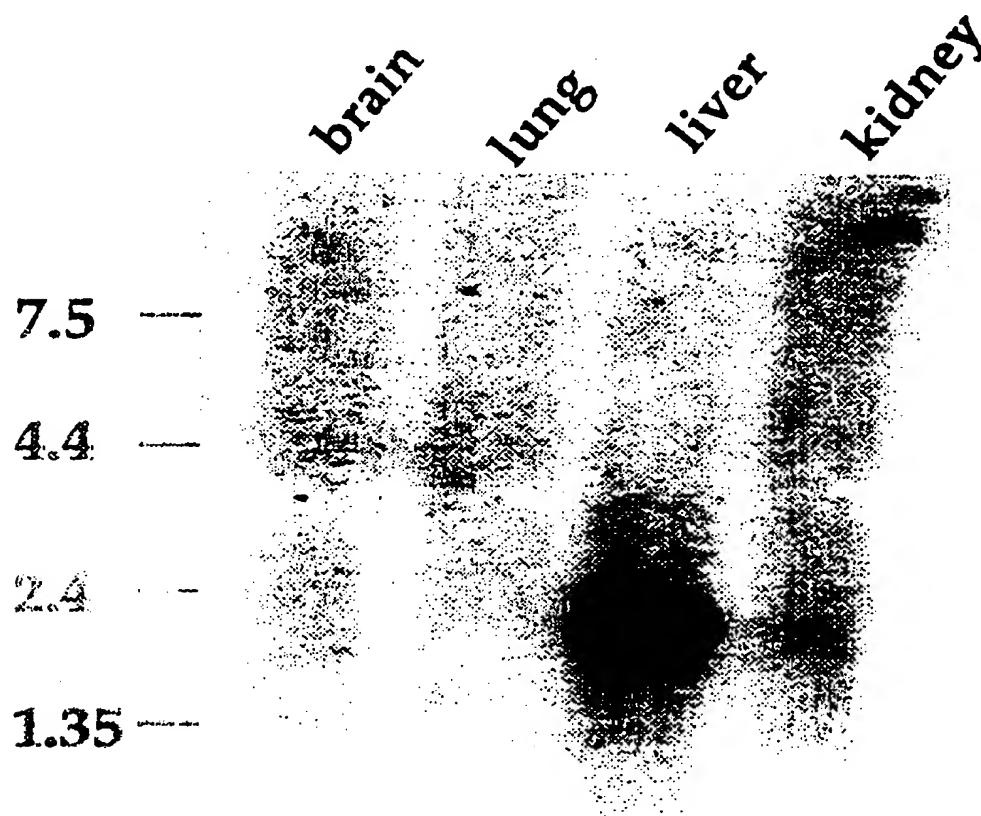


FIG. 8C

FIG. 8D

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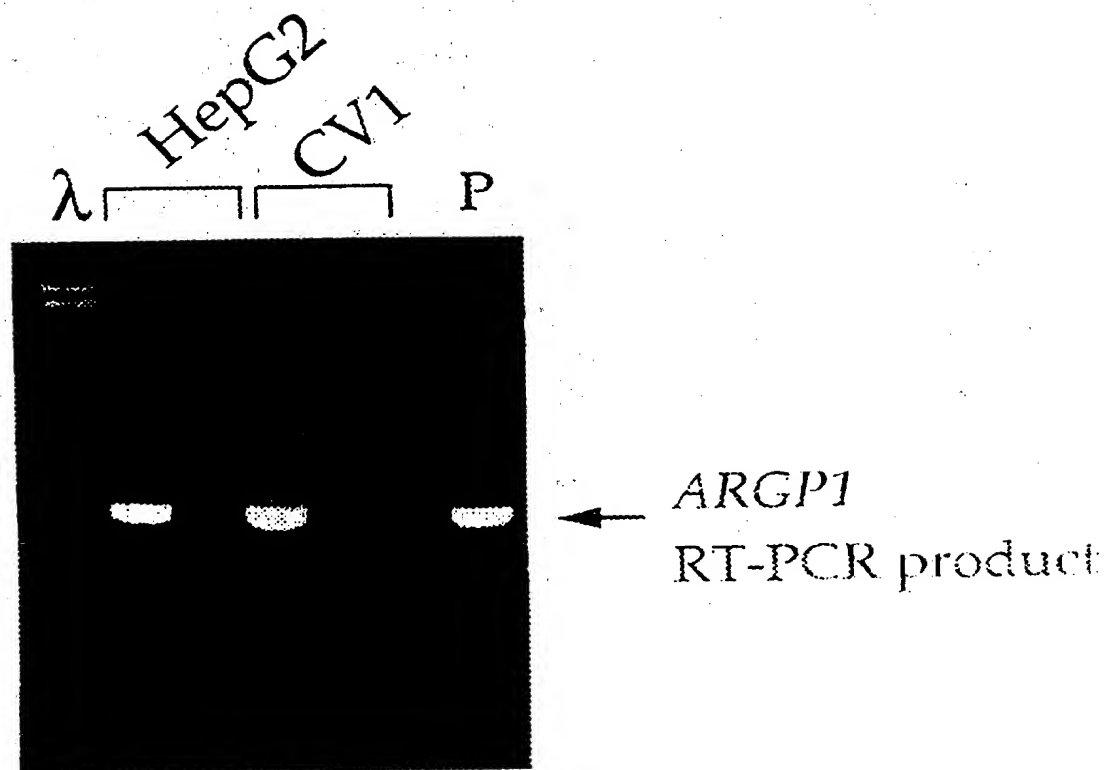
FIG. 9



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FIG. 10



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FIG. 11

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ARGP1      1 .....MSNYRGILNWCVVMLI 15
                .:.|.|.:. :.:.|
hACAT    101 EGEKNNHRAKDLRAPPEQGKIFIARRSLDELLEVDHIRTIVHMFIALLI 150

ARGP1      16 LSNARLFLENLIKYGILVDPIQVVSFLKDPHSWPAPCLVIAANVFAVAA 65
                | . :. :|. | || :. :|. : . . . :. :. :|.|. :
hACAT    151 LFILSTLVVDYIDEGRLVLEFSLLSYAFGKFPTVVWTTWIMFLSTFSVPY 200

ARGP1      66 FQVEKRLAVGALTEQA.....GLLLHVANLATILCFPAAVVLLVESIT 108
                | :.:. :. . :. : | :| : :. :. :|.|. :. :|. :. :.
hACAT    201 FLFQHWRTGYSKSSHPLIRSLFHGFLFMIFQIG.VLGFGPTYVVLAYTLP 249

ARGP1      109 PVGSLALMAHTILFLKPFYSYRDVNSWCRRARAKAASAGKKASSAAAPHT 158
                | :.:. :. :. :. :| : | :| . :. :. :|. | :. :. |
hACAT    250 PASRFIIIFEQIRFVMA.....HSFVRENVPRVLNSAKEKSSTVPIPT 293

ARGP1      159 VSYPDNLTyrDLYYFLFAPTLCYELNFPSPRIRKRFLLRILEMLFFTQ 208
                | . :. :| | | | | | | | | | | | | | | | | | | | | | |
hACAT    294 VN.....QYLYFLFAPTLIYRDSYPRNPTVRWGYVAMK.FAQVFGCF 334

ARGP1      209 LQVGLIQQWMVPTIQNSMKPFKDMDSRIIERLLKLAV.....PNHLIWL 253
                : | . | :. :. :. : | :. :. :. :|. | | | | | | | | | | | |
hACAT    335 FYVYYIFERLCAPL.....FRNIQEPFSARVLVLCVFNSILPGVLILF 378

ARGP1      254 IFFYWLFHSCNAVAELMQFGDREFYRDWWNSESVTYFWQWNIPVHKWC 303
                : | : :. :| : | | | | | | | | | | | | | | | | | | | | | |
hACAT    379 LTFFAFLHCWLNFAEMLRFGDRMFYKDWWNSTSYSNYYRTWNVVVDHWL 428

ARGP1      304 IRHFYKPMRLRGSSKWM..ARTGVFLASAFFHEYLVSVPLR.....MFRL 346
                . . | | :| : | :. :. | . :| | | | | | | | | | | | | | | |
hACAT    429 YYYAYKDFLWFFSKRFKSAAMLAVFAVSAVVHEYALAVCLSFFYPVLFVL 478

ARGP1      347 WAFTGMMAQIPLAWFVGRFFQGNYGNAAVWLSLIIGQPIAVLMYVHDYYV 396
                : | | | :. :. :| . . | . :| | | :| :. :. :| :. :| :. :|
hACAT    479 FMFFGM....AFNFIVNDSRKKPIWNVLMWTSFLGNGVLLCFYSQEWYA 524

ARGP1      397 LNYEAPAAEA..... 406
                . . : . .
hACAT    525 RRHCPLKNPTFLDYVRPRSWTCRYVF 550

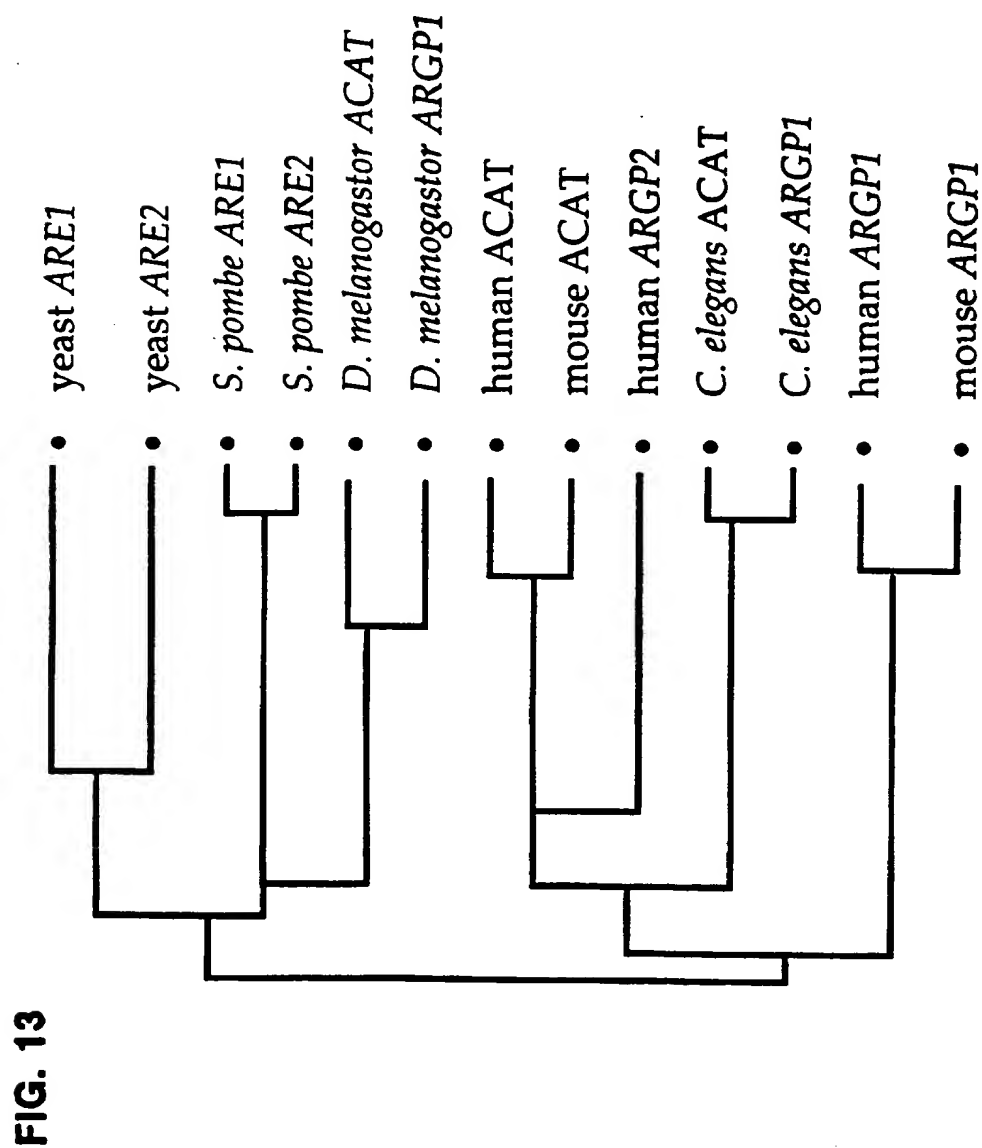
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FIG. 12

ARGP2	1M.FTPALGCVFYACFIVARFCVPVFA	24
hACAT	301	LFAPTLIYRDSYPRNPTVRWGYVAMKFAQVFCFFYVYIFERLCAPLFR	350
ARGP2	25	NMSREPFSTRALVFFILHATLPGIFMLLLIFFAFLHCWLNFAEMLRFGD	74
hACAT	351	NIKQEPFSARVLVLCVENSILPGVLILFLTFFAFLHCWLNFAEMLRFGD	400
ARGP2	75	RMFYRDWNNSTSFNSYRTWNVVHDWLYSVYQDGLRLLGARARGVAML	124
hACAT	401	RMFYKDWNNSTSYSNYRTWNVVHDWLYYAYKDFLWFFSKRFSAAAML	450
ARGP2	125	GVFLVSAVAHEYIFCFVLGFFYPVMLILFLVIEGMLNFMHMQRTGPWN	174
hACAT	451	AVFAVSADVHEYALAVCLSFYPVLFVLFMFFGMAFNFIVNDNRKKPIWN	500
ARGP2	175	VLMWTMLFLGQGIQVSLYCQEWYARRHCPLPQATFWGLVTPRSWSCHT..	222
hACAT	501	VLMWTSLFLGNGVLLCFYSQEWYARRHCPLKNPTFLDYVRPRSWTCRYVF	550

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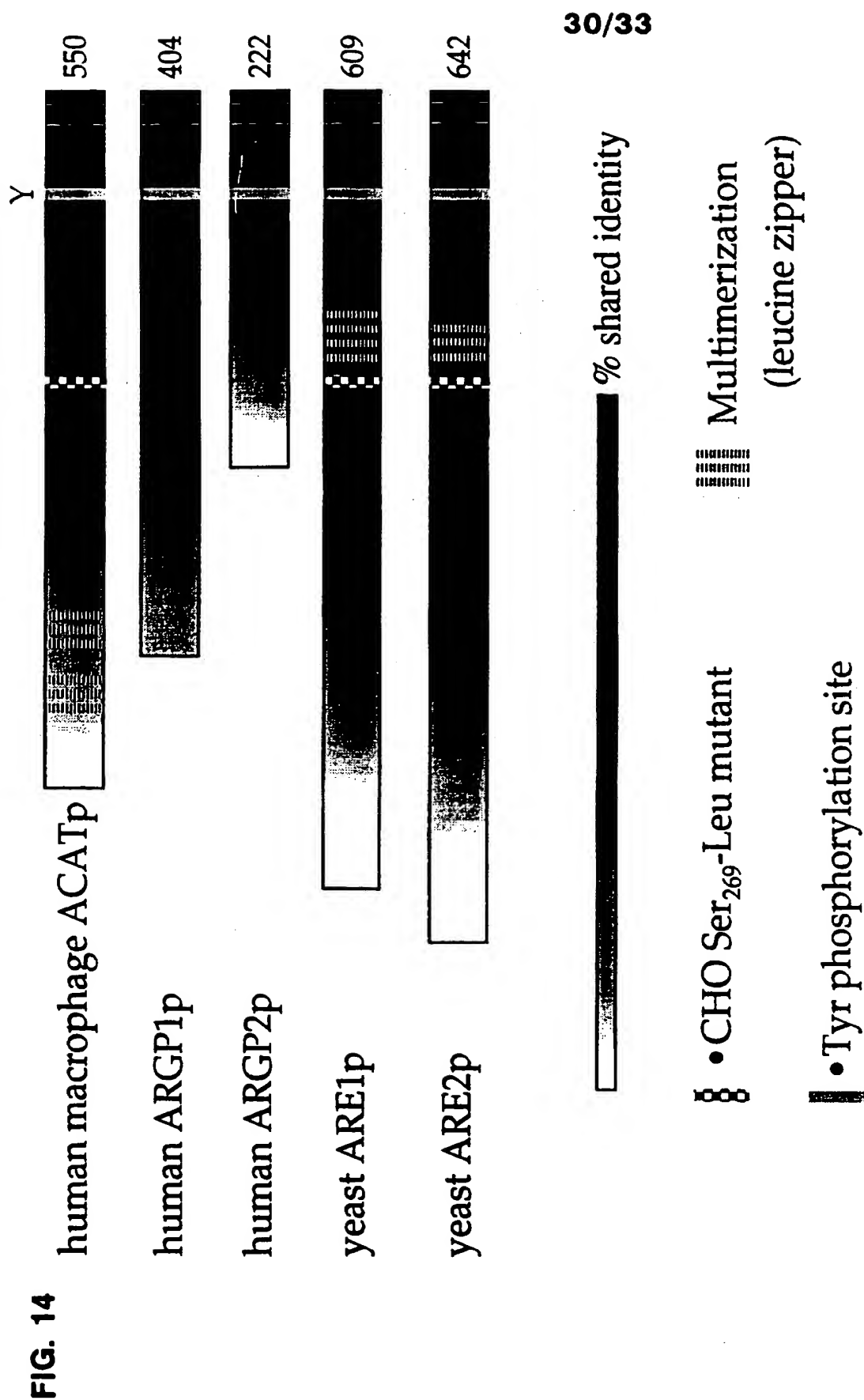


FIG. 15A

1	ATG	AGC	AAC	TAC	CGT	GGC	ATC	CTG	AAC	TGG	TGT	GTG	GTG	ATG	CTG	ATC	TTG	AGC	AAT	GCC	CGG	TTA	TTT	CTG	GAG	75
1	M	S	N	Y	R	G	I	L	N	W	C	V	V	M	L	I	L	S	N	A	R	L	F	L	E	25
76	AAC	CTC	ATC	AAG	TAT	GGC	ATC	CTG	GTG	GAC	CCC	ATC	CAG	GTG	GTT	TCT	CTG	TTC	CTG	AAG	GAT	CCC	CAT	AGC	TGG	150
26	N	L	I	K	Y	G	I	L	V	D	P	I	Q	V	V	S	L	F	L	K	D	P	H	S	W	50
151	CCC	GCC	CCA	TGC	CTG	GTT	ATT	GCG	GCC	AAT	GTC	TTT	GCT	GTG	GCT	GCA	TTC	CAG	GTT	GAG	AAG	CGC	CTG	GCG	GTG	225
51	P	A	P	C	L	V	I	A	A	N	V	F	A	V	A	A	F	Q	V	E	K	R	L	A	V	75
226	GGT	GCC	CTG	ACG	GAG	CAG	GCG	GGA	CTG	CTG	CTG	CAC	GTA	GCC	AAC	CTG	GCC	ACC	ATT	CTG	TGT	TTC	CCA	GCG	GCT	300
76	G	A	L	T	E	Q	A	G	L	L	L	H	V	A	N	L	A	T	I	L	C	F	P	A	A	100
301	GTG	GTC	TTA	CTG	GTT	GAG	TCT	ATC	ACT	CCA	GTG	GGC	TCC	CTG	CTG	GCG	CTG	ATG	GCG	CAC	ACC	ATC	CTC	TTC	CTC	375
101	V	V	L	L	V	E	S	I	T	P	V	G	S	L	L	A	L	M	A	H	T	I	L	F	L	125
376	AAG	CCC	TTC	TCC	TAC	CGC	GAC	GTC	AAC	TCA	TGG	TGC	CGC	AGG	GCC	AGG	GCC	AAG	GCT	GCC	TCT	GCA	GGG	AAG	AAG	450
126	K	P	F	S	Y	R	D	V	N	S	W	C	R	A	R	A	K	A	A	S	A	G	K	K	K	150
451	GCC	AGC	AGT	GCT	GCT	GGC	CCG	CAC	ACC	GTG	AGC	TAC	CCG	GAC	AAT	CTG	ACC	TAC	CGC	GAT	CTC	TAC	TAC	TTC	CTC	525
151	A	S	A	A	A	A	P	H	T	V	S	Y	P	D	N	L	T	Y	R	D	L	Y	Y	F	L	175
526	TTC	GCC	CCC	ACC	TTG	TGC	TAC	GAG	CTC	AAC	TTT	CCC	CGC	TCT	CCC	CGC	ATC	CGG	AAG	CGC	TTT	CTG	CTG	CGA	CGG	600
176	F	A	P	T	L	C	Y	E	L	N	F	P	R	S	P	R	I	R	K	R	F	L	L	R	R	200
601	ATC	CTT	GAG	ATG	CTG	TTC	TTC	ACC	CAG	CTC	CAG	GTG	GGG	CTG	ATC	CAG	TGG	ATG	GTG	CCC	ACC	ATC	CAG	AAC	675	
201	I	L	E	M	L	F	F	T	Q	L	Q	V	G	L	I	Q	Q	W	M	V	P	T	I	Q	N	225
676	TCC	ATG	AAG	CCC	TTC	AAG	GAC	ATG	GAC	TAC	TCA	CGC	ATC	ATC	GAG	CGC	CTC	CTG	AAG	CTG	GCG	GTC	CCC	AAT	CAC	750
226	S	H	K	P	F	K	O	M	D	Y	S	R	I	I	E	R	L	L	K	L	A	V	P	N	H	250

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FIG. 15B

751 CTC ATC TGG CTC ATC TTC TTC TAC TGG CTC TTC CAC TCC TGC CTG AAT GCC GTG GCT GAG CTC ATG CAG TTT GGA 825
 251 L I W L I F Y W L F H S C L N A V A E L M Q F G 275
 826 GAC CGG GAG TTC TAC CGG GAC TGG TGG AAC TCC GAG TCT GTC ACC TAC TTC TGG CAG AAC TGG AAC ATC CCT GTG 900
 276 D R E F Y R D W W N S E S V T Y F W Q N W N I P V 300
 901 CAC AAG TGG TGC ATC AGA CAC TTC TAC AAG CCC ATG CTT CGA CGG GGC AGC AGC AAG TGG ATG GCC AGG ACA GGG 975
 301 H K W C I R H F Y K P M L R R G S S K W M A R T G 325
 976 GTG TTC CTG GCC TGG GCT TTC CAC GAG TAC CTG GTG AGC GTC CCT CTG CGA ATG TTC CGC CTC TGG GCT TTC 1050
 326 V F L A S A F F H E Y L V S V P L R M F R L W A F 350
 1051 ACG GGC ATG ATG GCT CAG ATC CCA CTG GCC TGG TTC GTG GGC CGC TTT TTC CAG GGC AAC TAT GGC AAC GCA GCT 1125
 351 T G M A Q I P L A W F V G R F F Q G N Y G N A A 375
 1126 GTG TGG CTG CTC ATC ATC GGA CAG CCA ATA GCC GTC CTC ATG TAC GTC CAC GAC TAC TAC GTG CTC AAC TAT 1200
 376 V W L S L I I G Q P I A V L M Y V H D Y Y V L N Y 400
 1201 GAG GCC CCA CGG GCA GAG GCC TGA gctgcacctgaggccctggcttctcactgccacctcaaacccgctgccagagccacctctcctcta 1292
 401 E A P A A E A . 408
 1293 ggcctcgagtgctgggatgggctggctgcacagcatcctcctctggtcccgaggagcctctctgccctatggggctctgtctgcacccctcaggga 1392
 1393 tggcgacagcaggccagacacagctctgatgccagctgggagctctgtgacctgccccgggtccgagggtgtcaataaagtgtgtccagtgaggagaaa 1492
 1493 aaaaaaaaaaaaaattctgcggccgc 1521

FIG. 16

1 ATG TTT ACC CCA GCC CTG GGA TGT GTG TTC TAT GCC TGC TTC ATC GTG GCC CGC TTC TGT GTT CCT GTC TTT GCC 75
 1 M F T P A L G C V F Y A C F I V A R F C V P V F A 25
 76 AAC ATG AGC CGA GAG CCC TTC AGC ACC CGT GCC TTT GTG TTT TTT ATC CTG CAT GCC ACG TTG CCA GGC ATT TTC 150
 26 N M S R E P F S T R A L V F F I L H A T L P G I F 50
 151 ATG CTG CTG CTC ATC TTC TTT GCC TTC CTC TGC TGG CTG AAC GCC TTT GCC GAG ATG CTA CGA TTT GGA GAC 225
 51 M L L L I F A F A F L C W L N A F A E M L R F G D 75
 226 AGG ATG TTC TAC CGG GAC TGG TGG AAC TCA ACG TCC TTC TCC AAC TAC TAC CGC ACT TGG AAC GTG GTG CAT 300
 76 R M F Y R D W W N S T S F S N Y Y R T W N V V V V H 100
 301 GAC TGG CTG TAC AGC TAC GTG TAT CAG GAT GGG CTG CGG CTC CTT GGT GCC CGG GGC CGA GGG GTA GCC ATG CTG 375
 101 D W L Y S Y V Y Q D G L R L G A R A R G V A M L 125
 376 GGT GTG TTT CTG GTC TCC GCA GTG GCC CAC GAG TAT ATC TTC TGC TTC GTG CTG GGG TTC TTC TAT CCC GTC ATG 450
 126 G V F L V S A V A V A H E Y I F C F V L G F F Y P V M 150
 451 CTG ATA CTC TTC CTT GTC ATT GAA GGA ATG TTG AAC TTC ATG CAT GAC CAG CGC ACC GGC CGC GCA TGG AAC 525
 151 L I L F L V I E G M L N F M H D Q R T G P A W N 175
 526 GTG CTG ATG TGG ACC ATG CTG TTT CTA GGC CAG GGA ATC CAG GTC AGC CTG TAC TGC CAG GAG TGG TAC GCA CGG 600
 176 V L M W T M L F L G Q G I Q V S L Y C Q E W Y A R 200
 601 CGG CAC TGC CCC TTA CCC CAG GCA ACT TTC TGG GGG CTG GTG ACA CCT CGA TCT TGG TCC TGC CAT ACC TAG aggt 676
 201 R H C P L P Q A T F W G L V T P R S W S C H T 224
 677 cgggacagacgactgcccagacaccaccaagttctctgcctgcaaaacctggggaccaggactccctgtctgattccccaaatttggtctga 776
 777 gtcgaggcaacctgcacaaagacccccaccacaaaggaatgtgcaaggactgagatctgcagactgtgtggtaactgatcacagacctcagcatgggggtg 876
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 1077 cccttt 1082

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/09460**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) :C07H 21/04; C12N 15/11; C12P 21/02

US CL :536/23.2, 23.5; 435/70.1, 320.1, 325

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 536/23.2, 23.5; 435/70.1, 320.1, 325

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE, BIOSIS, CA, DERWENT

search terms: acylcoenzyme A: cholesterol acyltransferase

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ---	Expressed Sequence Tag (EST) database Accession Numbers R99213 (13 September 1995), R99214 (13 September 1995), H27149 (12 July 1995), N76754 (02 April 1996), H45923 (31 July 1995), H45924 (31 July 1995), H24791 (07 July 1995), Z39933 (21 September 1995), T79408 (15 March 1995), R48474 (18 May 1995), T79494 (15 March 1995), R48475 (18 May 1995), M79086 (26 May 1992), and W10786 (26 April 1996).	1-12 and 33 -----
Y		13-32
Y	US 5,484,727 A (CHANG et al.) 16 January 1996, cols. 1-5.	13-32

☐ Further documents are listed in the continuation of Box C.☐ See patent family annex.

* Special categories of cited documents:	*T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
E earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*&* document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means	
P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

19 AUGUST 1997

Date of mailing of the international search report

29 AUG 1997

Name and mailing address of the ISA/US
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Authorized officer

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Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US97/09460

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-33

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/09460

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claim(s) 1-33, drawn to acylcoenzyme A: cholesterol acyltransferase II and III nucleic acids and enzymes, and a first method of use comprising recombinant expression.

Group II, claim(s) 34-39, drawn to a second method of use comprising detecting a defect in esterification of sterol in a subject.

Group III, claim(s) 40, drawn to third method of use comprising treating a subject having an imbalance in sterol levels.

Group IV, claims 41-44, drawn to a fourth method of use comprising inhibiting wildtype acylcoenzyme A: cholesterol acyltransferase II or III in a subject.

Group V, claim 45, drawn to a fifth method of use comprising identifying a chemical compound which is capable of inhibiting acylcoenzyme A: cholesterol acyltransferase II or III in a subject.

Group VI, claims 46-48, drawn to an undefined pharmaceutical compound and methods of its use in treating a subject.

Group VII, claim 49, drawn to a transgenic, nonhuman mammal.

The inventions listed as Groups I-VII do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: PCT Rule 13.2 only provides for grouping together of a first product and a first method of use, rather than for multiple uses. Further, the compound and uses of group VI and the transgenic mammal of group VII lack the same or corresponding special technical feature as the other groups, which relate specifically to the acylcoenzyme A:cholesterol acyltransferase II or III sequence.

